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RATE OF FORMATION *IN VIVO* OF THE UNREACTIVATABLE FORM OF BRAIN CHOLINESTERASE IN CHICKENS GIVEN DDVP OR MALATHION*

ROBERT F. WITTER and THOMAS B. GAINES

Toxicology Section, Communicable Disease Center, United States Public Health Service, Atlanta, Ga., U.S.A.

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Abstract—The half-time for the conversion of the reversibly inhibited form of the dimethyl phosphorylated cholinesterase to the irreversibly inhibited form of this enzyme in the brains of chickens given DDVP or malathion has been found to be 2 hr. The same half-time is observed *in vitro* in brain homogenates inhibited with DDVP or malaoxon, the active form of malathion.

THE RATE of formation of the irreversibly inhibited form of cholinesterase from the initial reversibly inhibited enzyme has been shown to be dependent on the dialkoxy group which is attached to the phosphorylated enzyme.

Thus, *in vitro*, the rate of the conversion increased in the order: diethyl phosphate < dimethyl phosphate and diisopropyl phosphate.[1–4] *In vivo*, a similar transformation has been noted with the cholinesterases of rabbit erythrocytes,[5] mouse brain and erythrocytes,[6] rat brain and erythrocytes,[7] and sheep erythrocytes.[8] However, the rate of the conversion of the reactivatable to the unreactivatable form of cholinesterase *in vivo* has been measured only for the diethyl and diisopropyl phosphorylated enzyme of mouse brain and erythrocytes. The half-time for the transformation of the diisopropyl phosphate enzyme was 4 hr; that of the diethyl phosphorylated enzyme was 36 hr.[6] It is known that the rate of formation of the irreversible form of the dimethyl phosphorylated enzyme of rat brain and erythrocytes[4,7] and sheep erythrocytes[8] *in vivo* is quite rapid.

However, previous investigators have not been able to measure accurately the rate of transformation of the dimethyl phosphorylated cholinesterase to the unreactivatable form because this process was so fast that significant conversion of the inhibited enzyme took place during the assay period. In addition, the rate of spontaneous reactivation was so rapid that the process of formation of the irreversibly inhibited enzyme could not be separated from the spontaneous reactivation of the inhibited enzyme.[4,7,8]

In previous experiments it was found that the brain cholinesterase of chickens given a subcutaneous dose of either of the dimethyl phosphates, malathion or DDVP, remained 85 to 90 per cent inhibited for one day.[9] This appeared to be sufficient time of inhibition to permit measurement *in vivo* of the rate of the transformation of the

* DDVP is O,O-dimethyl 2,2-dichlorovinyl phosphate; malathion is O,O-dimethyl S-(1,2-dicarbethoxyethyl) phosphorodithioate.

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reversible to the irreversible form of cholinesterase uncomplicated by significant spontaneous reactivation of the enzyme. In addition, since malathion was given at 40 times the level of DDVP on a molar basis, it was possible to study the effect of this large difference in dosage on the rate of the transformation *in vivo*.

In these studies it was necessary to use a procedure that permitted a very rapid reactivation *in vitro* so that significant formation of the irreversibly inhibited enzyme did not take place during the reactivation. In preliminary experiments it was found that reactivation could take place very rapidly with an excess of 2-PAM (2-pyridine aldoxime methiodide),[10,11] and this procedure was used in the experiments reported in this paper.

EXPERIMENTAL

Sources of chemicals

The sources of most of the chemicals used and the method and rate of subcutaneous dosing of the chickens are given in another paper.[9] Acetylcholine perchlorate was obtained from K and K Laboratories,* Jamaica, N.Y.; 2-PAM was obtained from the Chemical Procurement Co., New York, N.Y.; malaoxon [O,O-dimethyl S-(1, 2-dicarbethoxyethyl) phosphorothioate] was a highly purified product of the American Cyanamid Co., New York 20, N.Y. With the exceptions listed below, all other methods are given in another publication.[9]

Measurement of cholinesterase activity

The cholinesterase activity of brain was determined with acetylcholine as the substrate in a manner designed to prevent reaction between enzyme and inhibitor during homogenization. This procedure was based on previous observations that the rate of the reaction between organic phosphorus compounds and cholinesterase is temperature dependent[12] and is decreased by the presence of acetylcholine.[12–17]

Three observations showed that the method employed was an assay of brain true cholinesterase. First of all, the hydrolysis of this choline ester was decreased when the concentration was raised above 0·01 M. Second, the rate of hydrolysis of butyrylcholine, a substrate for pseudocholinesterase of chicken brain,[18,19] was only 5 per cent that of acetylcholine. Third, acetyl-β-methylcholine was hydrolysed at 37·5 per cent the rate of acetylcholine. The ratio of the rates of hydrolysis of these two substrates is similar to that observed with other true cholinesterases.[20]

The assay of brain true cholinesterase was carried out in the following manner. The whole brain of a chicken was quickly removed, chilled, and homogenized at 0° with a motor-driven, all-glass homogenizer[21] in 4 volumes of solution which contained 0·02 M acetylcholine perchlorate, 0·095 M NaCl, 0·004 M KCl, 0·001 M KH_2PO_4, 0·001 M $MgCl_2$, and 0·02 M $NaHCO_3$. This solution was gassed 5 min with nitrogen–carbon dioxide (95/5) and kept in a stoppered bottle until it was used for the preparation of brain homogenate. The homogenate had a pH of 7·45. No more than 45 min elapsed between the time the chicken was killed and the time the assay of the homogenate was begun. A 0·1-ml portion of the ice-cold homogenate was placed in the main compartment of the Warburg flask that was chilled in an ice bath at 0° and

* Throughout this paper, the use of trade names and the names of suppliers is for identification only and does not constitute endorsement by the Public Health Service.

that contained 3 ml of ice-cold 0·02 M sodium bicarbonate, 0·001 M magnesium chloride, 0·095 M sodium chloride, 0·004 M potassium chloride, 0·001 M potassium acid phosphate, and 0·01 M acetylcholine perchlorate. The sodium bicarbonate solution had been pregassed with N_2/CO_2, 95/5, v/v. The flask was gassed with N_2/CO_2, 95/5, v/v on the manometer at 0° for 6 min, was transferred to the water bath at 25°, and readings were begun after 5 min. The manometer readings were taken at 5-min intervals for 30 min.

It was shown by incubation of 10% brain homogenate with inhibitor that the procedure just described would prevent reaction between brain cholinesterase and 33 and 80 times the I_{50} concentration of DDVP and of malaoxon, respectively, for 2 hr but not for 18 hr at 0°. Malaoxon is the active metabolite of malathion[22] and is responsible for the inhibition observed in homogenates of the brains of chickens given malathion. This procedure afforded 85 per cent protection against these concentrations of inhibitor which were in great excess over those required to produce 95 per cent inhibition of brain cholinesterase at 25° under conditions in which the I_{50} concentration was measured.

Reactivation of brain homogenates with 2-PAM

The following procedure was used in studies of reactivation *in vitro* of brain cholinesterase in (a) homogenates inhibited *in vitro* with DDVP or malaoxon, or (b) chickens given DDVP or malathion. One-tenth ml aliquots of ice-cold homogenate, prepared as described in the previous section with acetycholine at 0°, were added to duplicate ice-cold flasks. One flask (A) contained 0·01 M acetylcholine and the other reagents given in the previous section. The other flask (B) contained 0·01 M acetylcholine, the other reagents, and also 0·0018 M 2-PAM in the main compartment. The flasks were ice-cold when the homogenate was added, were kept in ice during the 6-min gassing period, and were then brought to 25° within 4 to 5 min in order to start the enzymatic hydrolysis of acetylcholine. Reactivation occurred during this time, since no greater extent of reactivation was observed in incubating the homogenates at 25° with 0·02 M 2-PAM for 1 hr. More consistent results were obtained with the addition of the homogenate to the main compartment of the flask at 0° than by incubation in the side arm at 25°. At the concentration of acetylcholine and 2-PAM employed, 2-PAM inhibited the cholinesterase of homogenates of normal chicken brain an average of 7 per cent.

The per cent reactivation (%R), or the per cent of the enzyme in the reactivatable form, was calculated from the following formula, proposed by Hobbiger:[5]

$$\%R = 100\left(\frac{\text{rate of reactivated flask B} - \text{rate of inhibited flask A}}{\text{rate of control C} - \text{rate of inhibited flask A}}\right)$$

In experiments in which the per cent reactivation was measured in homogenates of the brains of poisoned chickens, the control rate C was the mean cholinesterase activity of twelve chickens corrected for 7 per cent inhibition by 2-PAM. In studies in which homogenates were inhibited *in vitro*, the control rate C was the rate of hydrolysis of acetylcholine in the presence of 2-PAM by the uninhibited homogenate from the same chicken.

This procedure was capable of reactivating 80 to 85 per cent of the cholinesterase in brain homogenates that had been inhibited by incubation at 25° for 1 hr with as high

as 1,000 times the I_{50} concentrations of DDVP or malaoxon. In these experiments *in vitro*, acetylcholine was omitted from the homogenization medium in order to facilitate the inhibition of the enzyme by malaoxon or DDVP. Reactivation of the inhibited cholinesterase by 2-PAM under the conditions of these experiments was not influenced by the presence of acetylcholine, and homogenates prepared from the same brain of a normal chicken had the same enzymatic activity in the absence or presence of acetylcholine in the homogenizing medium.

Measurement of pI_{50} *value*

The pI_{50} value of malaoxon or DDVP in chicken brain homogenates was determined in the following manner. A 0·2-ml portion of a 10% homogenate was incubated in the side arm of the Warburg flask at 25° in a N_2/CO_2, 95/5, v/v atmosphere with a solution containing 0·02 M sodium chloride and the other salts mentioned in the description of the cholinesterase assay except the acetylcholine which was also omitted from the solution used for homogenization. After 1 hr the contents of the side arm were dumped into the main compartment, and cholinesterase activity was measured with 0·01 M acetylcholine as previously described. Preliminary experiments indicated that 45 min at 25° was sufficient time to permit complete reaction between inhibitor and enzyme. The pI_{50} value was then calculated in the usual manner from a plot of pI versus per cent inhibition of the homogenate.

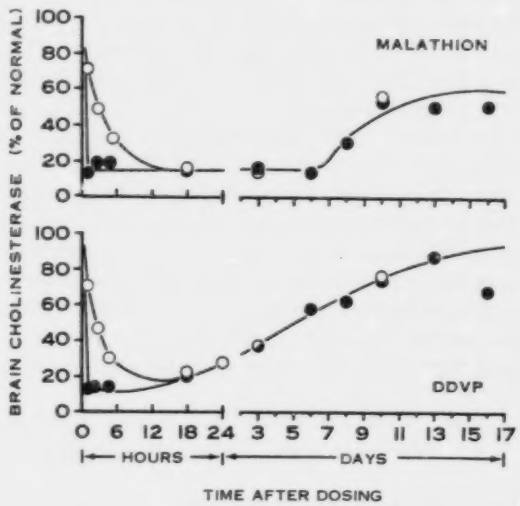

FIG. 1. Reactivation *in vitro* with 2-PAM of brain cholinesterase of chickens dosed with DDVP or malathion. In this experiment one group of chickens was dosed with malathion and the other with DDVP. At various time intervals fowls were sacrificed, and the brain true cholinesterase was determined as described in the experimental section. The ability of the inhibited enzyme to be reactivated was estimated in a duplicate flask to which 2-PAM was added according to the procedure described. Each point represents the average of the analysis of the brains of two chickens; ● represents per cent activity in the absence of 2-PAM; ○ represents the activity of the corresponding homogenates to which 2-PAM was added.

RESULTS AND DISCUSSION

The results of the studies of the rate of formation of the irreversible form of brain true cholinesterase in chickens dosed subcutaneously with 1,000 mg of malathion or 16 mg of DDVP/kg are presented in Figs. 1 and 2. It can be seen that the brain cholinesterase of chickens poisoned with either of these compounds was depressed to minimal value, 16 to 17 per cent of the control level, and remained inhibited for 18 hr. After that time, in confirmation of previous results,[9] the enzyme activity began to recover with DDVP but remained inhibited for 6 days with malathion. As shown in another paper,[9] the relatively rapid recovery of brain cholinesterase after DDVP is due to synthesis of new enzyme; retention of inhibitor in the body accounts, in part at least, for the slow recovery of brain cholinesterase after dosing with malathion.

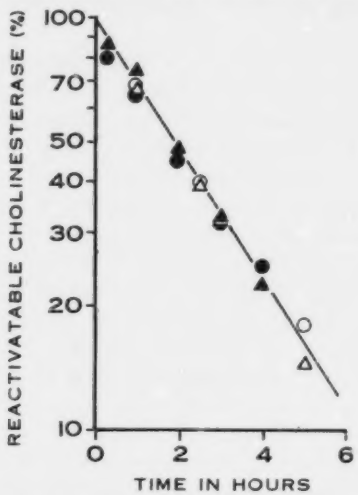

FIG. 2. The rate of disappearance of reactivatable brain cholinesterase *in vivo* was determined from the data on which the curves in Fig. 1 are based. The per cent reactivatable enzyme was calculated with these data according to the formula in the experimental section.

The rate of disappearance of reactivatable brain cholinesterase was measured *in vitro* in the following way. The homogenate containing no acetylcholine but in bicarbonate buffer (pH 7·4) in a N_2/CO_2 atmosphere was equilibrated at 40° for 10 min, and then sufficient inhibitor was added to bring its concentration to 1,000 times the I_{50} level. A control homogenate diluted with water also was run. At various intervals, 0·1-ml aliquots of the homogenates were assayed for cholinesterase activity in the presence and absence of 2-PAM. The details of the experimental procedure and the calculation of the per cent reactivatable enzyme are presented in the experimental section. The enzyme was inhibited 95 per cent with the concentration of inhibitor used and in the absence of 2-PAM did not increase in activity more than 5 per cent during the 4-hr incubation at 40°. ○, DDVP *in vivo*; ●, DDVP *in vitro*; △, malathion *in vivo*; ▲, malaoxon *in vitro*.

The ability of brain homogenates from chickens given DDVP or malathion to be reactivated with 2-PAM rapidly disappeared. Only 5 per cent of the enzyme could be reactivated after 18 hr. A plot of the logarithm of per cent reactivation with 2-PAM against time (Fig. 2) showed that the half-time for the disappearance of the reactivatable dimethyl phosphorylated cholinesterase was 2 hr for chickens dosed with either DDVP or malathion, despite the large difference in dosage between the two compounds. The

same value of 2 hr was found for the half-time of disappearance of the reactivatable form of brain cholinesterase in homogenates inhibited with 1,000 times the I_{50} concentration of DDVP or malaoxon, respectively. The I_{50} concentrations of these inhibitors were $1{\cdot}25 \times 10^{-7}$ M and $1{\cdot}8 \times 10^{-8}$ M for DDVP and malaoxon, respectively.

The rate of regeneration of brain cholinesterase *in vivo* is governed by several opposing factors. Inhibition with the active form of the inhibitor and the formation of the irreversible form of cholinesterase from the reversible form act to keep the enzyme inhibited. On the other hand, spontaneous reactivation of the inhibited cholinesterase and the synthesis of new cholinesterase molecules tend to reactivate the enzyme. In the present experiments over the period of 18 hr, synthesis *de novo* of new enzyme could not contribute significantly to the rate of regeneration of cholinesterase.

The same factors also govern the rate of regeneration of cholinesterase *in vitro*. Vandekar and Heath[7] have found *in vitro* that the rate of spontaneous reactivation of the dimethyl phosphorylated brain cholinesterase, such as that formed from malaoxon or DDVP, is quite fast; the half-time for this reaction is 1·3 hr at 37°. In the present studies *in vitro*, the brain cholinestersae remained inhibited during the experiment since, as is shown in the legend of Fig. 2, a large excess of inhibitor was present, and less than 5 per cent of the enzyme was spontaneously reactivated. Therefore, in these experiments the rate of inhibition *in vitro* of brain cholinesterase must have been greater than the rate of spontaneous reactivation of the dimethyl phosphorylated enzyme. Since the latter reaction, according to the results of Vandekar and Heath[7] cited above, is faster than the rate of disappearance of the reactivatable enzyme as determined *in vitro* in the present experiments (i.e., half-time 2 hr), the rate of conversion of the reversible form of cholinesterase to the irreversible form of the dimethyl phosphorylated enzyme must be the rate-limiting step in the disappearance of the reactivatable enzyme. Thus, the rate of disappearance of the reactivatable form of the inhibited cholinesterase measured in the present experiments is the same as the rate of formation of the irreversible form of the dimethyl phosphorylated enzyme from the reversible form of the inhibited cholinesterase.

The brain cholinesterase of chickens poisoned with DDVP or malathion was inhibited to rates 16 or 17 per cent of normal within 1 hr of dosing and remained inhibited for at least 18 hr. Thus, the rate of inhibition *in vivo* must have been greater than the rate of spontaneous reactivation of the dimethyl phosphorylated enzyme.

The rate of disappearance of the reactivatable form of the dimethyl phosphorylated enzyme was independent of the dosage of the inhibitor administered over a 40-fold range and was the same *in vivo* as *in vitro*. It therefore seems highly probably that the rate-limiting step in the disappearance of the reactivatable form of the dimethyl phosphorylated enzyme *in vivo* also is the rate of conversion of the reactivatable form of the inhibited cholinesterase to the unreactivatable form of this enzyme. Thus the rate of disappearance of the reactivatable form of the inhibited cholinesterase *in vivo* also is the same as the rate of formation of the irreversible form of the dimethyl phosphorylated enzyme from the reversible form of the inhibited cholinesterase.

As far as we are aware, this is the first time that the rate of formation of the irreversible form from the reversibly inhibited dimethyl phosphorylated enzyme has been measured.

The results of the present experiments, when combined with those of Hobbiger,[6] indicate that the rate of formation of the irreversibly inhibited dialkyl phosphorylated enzymes of brain increases in the order: diethyl phosphate < diisopropyl phosphate < dimethyl phosphate.

The methods employed for the protection of the cholinesterase in the homogenate from the reaction with inhibitor previously separated spatially from the enzyme, and for the reactivation of the enzyme with 2-PAM, should be of value to others who wish to study the inhibition and reactivation of cholinesterase *in vivo*. However, in other applications of these procedures, it will be necessary to prove that they will work with each new source of cholinesterase and with other organic phosphorus compounds.

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CHAPTER III

SAFE USE OF PESTICIDES AS RELATED TO HUMAN HEALTH

Homer R. Wolfe, Acting Chief
Western Pesticide Research Laboratory
Pesticides Program, Food and Drug Administration
Wenatchee, Washington

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PESTICIDES PROGRAM
National Communicable
Disease Center
Atlanta, Ga. 30333

Anyone who works with chemical pesticides must bear in mind that a certain amount of hazard accompanies the use of such materials. Each year in Washington State there are a number of illnesses, and in some instances deaths, attributable to pesticides. These cases are usually a result of gross carelessness or accident. A realization of the hazards inherent in pesticide materials, however, should not discourage their use but rather should stimulate safer handling. The following information concerning some of the more widely used pesticides, as well as some suggestions and precautions to follow when using such compounds, should be helpful to anyone working with pesticides.

Information Concerning Some of the More Extensively Used Pesticides: The more extensively used modern synthetic insecticides are the organic phosphorus, chlorinated hydrocarbon, and carbamate compounds. Generally, the acute toxicity of the organic phosphorus group is somewhat greater than that of the chlorinated hydrocarbon or the carbamate compounds. However, the chlorinated hydrocarbon compounds, due to their greater stability, present more of a residue problem.

The effect of the organic phosphorus compounds on the human body is through the nervous system. The organic phosphorus materials inhibit the enzyme cholinesterase, which functions in the transmission of nerve impulses. Among the more hazardous of the organic phosphorus compounds are parathion, TEPP, mevinphos (Phosdrin), demeton (Systox), disulfoton (Di-Syston), schradan (OMPA), phorate (Thimet), and Zinophos.

The antidote of choice in the treatment of organic phosphorus poisoning has been atropine, which acts as an antagonist to the nervous sytem effects of these compounds. A more specific antidote known as 2-PAM, which actually breaks down the combination between the enzyme and the organic phosphorus molecule, is also available. 3/

The exact physiologic mechanism by which the chlorinated hydrocarbon compounds exert their toxic action is not known. However, their primary effect is on the nervous system. The chlorinated hydrocarbons are fat-soluble and tend to accumulate in the body fat of animals exposed to them. Lactating animals that have been exposed excrete the chlorinated hydrocarbon materials or their derivatives in milk.

Among the more hazardous of the chlorinated hydrocarbon compounds are aldrin, dieldrin, and endrin.

The physiologic effect of the carbamate insecticides, as with

organic phosphorus compounds, involves inhibition of the enzyme cholinesterase. However, the effects of carbamate exposure tend to be less severe and not as long-lasting as the symptoms following organic phosphorus intoxication. The most widely used carbamate pesticide is carbaryl (Sevin), which is of low toxicity to mammals.

In general, the herbicides and fungicides are less toxic than many of the other types of pesticides. In Europe, a dinitro-type compound (DNOC) used for weed control has caused illnesses. 1/ Under the conditions of use in this country, however, tests have indicated that there is no significant hazard associated with the application of dinitro compounds for weed control. 4/ This conclusion has been corroborated by use experience.

One of the most widely used herbicides is 2,4-D. Use experience with 2,4-D has generally been good from the standpoint of human health. In cases of extensive respiratory exposure or accidental ingestion of commercial formulations of 2,4-D herbicides, the aliphatic or aromatic solvents used may cause certain poisoning symptoms characteristic of the solvents but not of 2,4-D.

The arsenical herbicides deserve special mention. While they are not particularly hazardous by skin contact, the arsenical herbicides have had a poor safety record. For example, in 1956, arsenic compounds were responsible for 54 of the total of 152 deaths (36%) caused by pesticides in that year in the United States. 2/

With the exception of mercury compounds, most common fungicides are less toxic than DDT. Acute poisoning by organic mercury compounds has been reported infrequently in man, although such poisoning has occurred. However, there have been many cases of chronic poisoning involving both inorganic and organic mercury. 3/ Most chronic cases have been associated with repeated exposure in connection with the manufacture of organic mercury compounds, their use for treating seed, or the eating of treated seed.

Certain fungicides, particularly ziram, may occasionally cause local irritations or dermatitis; however, these cases are usually not severe.

Safety Precautions to Follow When Using Pesticides: In nearly every case of human death from pesticides, accident or misuse of a chemical or plant poison has been involved. Follow these safety precautions when using pesticides:

General.

1. KEEP YOUR PESTICIDE STORAGE SHED OR ROOM LOCKED. Pesticides should be stored in the ORIGINAL, labeled containers and in a dry, well-ventilated room or building away from human food or animal feed AND OUT OF THE REACH OF CHILDREN.

2. Be sure that those who work with you are aware of the hazard involved when using pesticides. Never work alone under conditions where you could not receive help promptly in case of an accident.

3. If you are in the pesticide application business, make sure that your physician knows the types of compounds you are using. If he

should provide you with a supply of atropine tablets for organic phosphorus poisoning, DO NOT take them before definite symptoms occur. If you ever take atropine tablets, call your physician as soon afterward as possible. KEEP ATROPINE TABLETS AWAY FROM CHILDREN.

Before Application.

1. READ THE LABEL each time you use a pesticide - no matter how often you have used it and no matter how well you think you know the instructions - and FOLLOW THE LABEL DIRECTIONS EXACTLY. ALSO, read and heed the warning statements found on the label.

2. When handling, mixing, or applying pesticides, avoid inhaling dust and fumes and avoid getting materials on the skin. If the concentrated form of a toxic pesticide comes in contact with the skin, stop work, and wash the contaminated area thoroughly. Remove contaminated clothing and make sure it is thoroughly cleaned.

3. Mix pesticides according to directions and apply at the recommended rate. Use the least toxic compounds that will do the job well.

4. Do not smoke, chew tobacco, or eat while mixing or applying pesticides. When mixing and using inflammable chemicals be especially careful to avoid fire hazards.

During Application.

1. Wear protective clothing (preferably water-repellent), such as goggles, gloves, aprons, respirators, and masks when so directed by the label. When respirators are required, during application of pesticide compounds, they should be approved by the USDA for the material in question. The filters should be changed each 8 hours of appreciable exposure, and more often if pesticide odor is detected through the mask. Gas masks with cannister-type filters should be used when high concentrations of toxic vapors are present or when there is prolonged exposure in confined spaces.

2. When spraying around dwellings warn the occupants to keep children out of the area, preferably for a period of at least 48 hours, or until the spray has completely dried on the foliage, grass or other sprayed surfaces. Make sure that children's toys and containers in the yard to not retain spray.

3. If concentrated pesticide is spilled on the ground, remove and bury the contaminated soil. This is especially important in areas where children may play.

4. If outdoors, do not spray into the wind; if indoors, work in a well ventilated area, to avoid inhalation of fumes.

5. In applying pesticides to food plants and crops: (a) use the proper dose recommended for the purpose, and (b) allow the full recommended time between applying the pesticide and harvesting the crop, to avoid having a harmful amount of pesticides remaining on food to be eaten. Do not plant food crops near ornamental plants which are to be sprayed.

After Application.

1. Get rid of used pesticide containers in a way that will not leave a hazard to people - particularly children - or to animals. Containers and waste pesticides should be buried under at least 18 inches of soil in a location carefully selected to insure that no surface or subsurface water will become contaminated. Metal drums should be thoroughly rinsed and punctured. If combustible pesticide containers are burned, exercise extreme caution and avoid the smoke.

2. Wash hands thoroughly after using pesticides and before eating or smoking.

3. Change clothing after each day's operations and bathe thoroughly.

Watch for These Symptoms.

Most pesticides easily enter the body through contact with skin, as well as through the mouth and throat and through breathing. Repeated exposures to such compounds may increase susceptibility to poisoning even though no immediate symptoms are produced. The initial symptoms of organic phosphorus poisoning are giddiness, headache, nausea, vomiting, excessive sweating, and tightness of the chest. These are followed by, or accompanied by, blurring of vision, diarrhea, excessive salivation, watering of the eyes, twitching of muscles, especially in the eyelids, and mental confusion. One of the most characteristic signs is constriction of the pupils, but this may be preceded by dilation. Late signs are fluid in the chest, convulsions, coma, loss of urinary or bowel control, and respiratory failure.

The symptoms of poisoning by the chlorinated hydrocarbon insecticides, such as dieldrin and endrin, are primarily due to their effect on the nervous system and include hyper-excitability, trembling or shaking and convulsions. General symptoms are a feeling of illness or discomfort, headache, fatigue, and possible lack of appetite and weight loss.

What to do in Case of Poisoning.

1. In severe cases of organic phosphorus poisoning, breathing may become labored or stop. In such a situation ARTIFICIAL RESPIRATION IS THE MOST IMPORTANT FIRST AID.

2. If contamination of skin or clothing is involved, decontaminate the patient by removing his clothing and bathing the contaminated skin areas with generous amounts of soap and water.

3. If poisoning has resulted from exposure to a contaminated atmosphere, remove the patient to fresh air.

4. If the insecticide has been swallowed and the patient has not vomited, induce vomiting by giving a tablespoonful of salt dissolved in one-half glass of warm water. Never try to give anything by mouth to an unconscious patient. Never induce vomiting when a caustic poisoning or organic solvent such as kerosene has been ingested.

5. If pesticide material has gotten into the eyes, wash them

immediately with flowing water.

6. Make the patient lie down and keep him warm.

7. Take the patient to a physician or hospital as soon as possible. Be prepared to give artificial respiration on the way.

8. Provide the physician with information from the label. Write down a list of ingredients for him or take along the entire container.

9. Where pesticide exposure is involved, do not allow the patient to return to work until advised by a physician that it is safe to do so.

In summary, both research and use experience with pesticides indicate that the very real hazard of certain pesticidal compounds requires appropriate precautions on the part of those using them--- but that approved pesticides can be used safely provided recommended precautions are followed. Thus, it is of primary impor ance to read and follow the precautions on the label.

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GAS CHROMATOGRAPHIC ANALYSIS OF PENTACHLOROPHENOL IN HUMAN URINE BY FORMATION OF ALKYL ETHERS

Morris Cranmer and Joseph Freal

Primate Research Branch, Division of Pesticides, Food and Drug Administration, Consumer Protection and Environmental Health Service, Perrine, Florida 33157

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PENTACHLOROPHENOL (PCP) is an herbicide, defoliant, and antimicrobic chemical used throughout the United States as a preservative agent for many products. Pentachlorophenol seems to be present everywhere, appearing in municipal water supplies, wells, paints, wood and paper products, and in urine of every person now being examined (1). The ubiquity of human exposure to this potentially dangerous compound has prompted concern in the field of public health (1-6). This interest has been stimulated by several recent industrial (3, 4) and public (5, 6) intoxications which resulted in fatalities.

This report describes a simple and rapid method for the determination of PCP based upon its conversion to any one or more of seven possible alkyl ethers. PCP ethers are detected and quantitated by electron capture gas chromatography and their identity is confirmed by p-values. Data are presented for seven GLC columns and three p-value solvent systems.

Materials and Methods

Apparatus:

A Micro-Tek MT-220 gas chromatograph, with tritium electron capture detector, was used. Inlet temperature was 220°C, column temperature 180°C, and nitrogen flow 80 ml/min. Gas chromatographic columns were U-shaped glass, 6' x 1/4". Glassware included a Kuderna-Danish evaporative-concentrator (Kontes K-569000); 15 and 1 ml test tubes; 15 and 5 ml ground glass-stoppered test tubes; 5, 0.4, and 0.1 ml pipettes.

Reagents:

Sodium hydroxide and hydrochloric acid, both analytical reagents. N-Methyl-N'-nitro-N-nitrosoguanidine, N-ethyl-N'-nitro-N-nitrosoguanidine, N'-nitro-N-nitroso-N-propylguanidine, N-isobutyl-N'-nitro-N-nitrosoguanidine, N-n-butyl-N'-nitro-N-nitrosoguanidine, N-isoamyl-N'-nitro-N-nitrosoguanidine, N-amyl-N'-nitro-N-nitrosoguanidine (Aldrich). Hexane, methanol, acetonitrile, dimethylformamide (Mallinckrodt, Nanograde).

Partition and Extraction:

Five ml of urine is transferred to a 15 ml ground glass-stoppered centrifuge tube. The urine sample is made alkaline by addition of 0.3 ml of 0.4 N NaOH. Five ml of hexane is added to and mixed with the alkaline urine sample. The layers are separated by centrifugation and the hexane layer is discarded. The alkaline urine is then made acidic by addition of 0.2 ml of concentrated HCl. The acidic urine is extracted by thorough mixing with exactly 2 ml of hexane. The sample is centrifuged and an aliquot of the hexane layer is then ready for derivatization.

Diazoalkane Generation:

(Use extreme caution; see Discussion.) Five ml of 20% NaOH is added to a 15 ml test tube. A 3 ml volume of hexane is placed on the 20% NaOH solution. One hundred mg of the nitrosoguanidine reagent is slowly added to the hexane in 10 mg increments (use a hood with a good draft). The reagent is ready for use after bubbles of the excess diazoalkane gas are no longer observed being evolved from the hexane solution.

Alkylation of PCP:

An aliquot, 0.4 ml, of the hexane extract of the acidic urine is pipetted into a 5 ml ground glass-stoppered test tube and then 0.1 ml of the alkylating reagent in hexane is added. The solution of diazoalkane plus hexane extract is mixed and allowed to stand. After 20 min, or a longer interval if

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convenient, 1 ml of 20% water in methanol is added to the reaction mixture and the two phases are mixed thoroughly. After phase separation, the hexane layer is ready for gas chromatographic and p-value examinations.

Gas Chromatographic Determination:

An aliquot, preferably 5 µl, of the alkylated PCP in hexane is injected into a gas chromatograph equipped with an electron capture detector and a suitable column (see Table I). Peaks are compared to peaks produced by injecting the same volume of standard PCP-ethers. Larger volumes (up to 20 µl) may be injected if similar volumes of diluted standards are used for quantitation. For peaks that are off scale, a smaller aliquot is taken or the sample is diluted.

If it is necessary to concentrate the sample, the organic solvent solution of PCP or PCP-ether is pipetted into an evaporative-concentrator tube. A two-bubble micro Snyder column is added and the tube is heated with steam, avoiding bumping and splashing, until the desired volume is obtained (7).

TABLE I

Retention Times of PCP-Ethers Relative to Aldrin[a]

PCP Ether	10% Apiezon L 80-100 Gas Chrom Q	3% DEGS, 80-100 Gas Chrom P	4% SE-30+ 6% QF-1, 80-100 Anachrom ABS	2% QF-1+ 3% OV-1, 80-100 Chrom W	5% QF-1, 80-100 Gas Chrom Q	3% OV-1 on 100-120 Chrom W	1.5% OV-17+ 1.95% QF-1, 100-120 Chrom W
Methyl	0.51	0.62	0.64	0.47	0.56	0.47	0.45
Ethyl	0.66	0.66	0.72	0.55	0.64	0.57	0.47
Propyl	1.00	0.73	0.95	0.73	0.80	0.78	0.67
Isobutyl	1.24	0.77	1.11	0.86	0.92	0.95	0.75
Butyl	1.41	0.91	1.25	0.99	0.97	1.10	1.04
Isoamyl	1.81	1.03	1.50	1.20	1.20	1.31	1.13
Amyl	2.20	1.11	1.72	1.39	1.31	1.55	1.41

[a]GLC conditions: 6' x 1/4" glass U-tube columns, 80 ml/minute nitrogen, and 180°C column temperature.

Determination of p-Values:

Individual volumes of acetonitrile, methanol, and dimethylformamide are equilibrated 1:1 with hexane at room temperature for 24 hours. A 0.1 ml aliquot of the hexane solution containing PCP-ether is pipetted into a 1 ml test tube and 0.1 ml of an equilibrated solvent is added. The two phases are thoroughly equilibrated by mixing 1 minute on a vortex mixer. After phase separation the upper hexane layer is ready for GLC. The p-value is the concentration of PCP ether in the hexane phase after partition divided by the concentration in the hexane before partition (see Table II).

TABLE II

p-Values of PCP-Ethers Determined by Single Distributions Between Immiscible Phases at 25° C

PCP Ether	Acetonitrile: Hexane	Methanol: Hexane	Dimethylformamide: Hexane
Methyl[a]	0.62	0.61	0.44
Ethyl[a]	0.71	0.63	0.52
Propyl[a]	0.73	0.69	0.57
Isobutyl[b]	0.81	0.69	0.68
Butyl[b]	0.80	0.68	0.65
Isoamyl[b]	0.82	0.71	0.68
Amyl[b]	0.80	0.63	0.65

[a] p-value consistently reproduced within 0.03.

[b] p-value consistently reproduced within 0.02.

Results

Urine samples from individuals representative of the general population and from occupationally exposed individuals were analyzed in replicate, using this procedure. Replicates varied less than ±5% from their means over the range of 2.2 to 265 ppb PCP (Table III). The standard deviation decreased as the concentration of PCP increased.

TABLE III

Concentration Range of Pentachlorophenol in Human Urine

Sample	Origin	Replicate Range[a], ppb	Mean[b], ppb
1	General Population	2.2 - 2.3	2.2
2	General Population	2.7 - 2.9	2.8
3	General Population	2.9 - 3.0	2.9
4	General Population	5.0 - 5.3	5.1
5	General Population	5.2 - 5.5	5.3
6	General Population	10.6 - 11.2	10.8
7	Carpenter	22.2 - 25.5	24.1
8	Boat Builder	55.0 - 60.1	57.3
9	Sprayman	131 - 136	133
10	Sprayman	259 - 270	265

[a]Four replicates of each sample.

[b]Variation of the method was ±5%.

Discussion

A number of precautions must be observed in using this procedure. All reagents, including the water used to prepare the NaOH, HCl, and MeOH solutions, must be extracted with hexane before use as they may be contaminated with PCP or other materials which interfere with the analysis. Deionized water is superior to distilled water with respect to the absence of PCP. Glassware should be washed with a dilute NaOH solution followed by deionized water and acetone rinses. Care should be taken not to allow contact between wooden or paper materials and glassware because peg boards and several brands of absorbent paper products have been found to contain PCP.

Preliminary extraction of the alkaline urine samples removes many interfering compounds that would subsequently be extracted from the acidic urine solution, including the neutral chlorinated hydrocarbons normally present in human urine (8). The volume of urine sampled and the amounts of acid and base

added have been selected to allow for urine with different pH values while excluding the largest amount of interfering material. It was found, as reported by Bevenue (9), that a more severe treatment of the urine before extraction, i.e., refluxing with concentrated acid, does not result in increased extraction of PCP. Also, additional extractions or extraction with a larger volume is unnecessary, because a single hexane extraction of acidified urine yields 90% of the PCP present. Five ml of urine is a volume convenient to manipulate; however, larger or smaller volumes of urine can be analyzed if the relative proportion of reagents to sample is maintained. If the suggested volumes are used, calculation of results is simplified because 1/2 the number of picograms of PCP derivative per µl injected is equal to ppb of PCP present in the urine.

Hexane was chosen as the solvent for the extraction and alkylation reaction because it extracts the fewest interfering substances from urine while acting as an efficient extractant for PCP, because it can be purchased commercially in an ultrapure form, and because it produces very little response with an electron capture detector.

Caution must be used when handling the skin irritant diazoalkane reagents since both the reagents and the diazoalkane gases are extremely toxic and potentially explosive (10). Diazoalkane generation should be carried out in a hood; use of disposable gloves is desirable and manufacturers' recommendations for storage and handling should be closely adhered to. Diazoalkane solutions should not be pipetted by mouth. It is suggested that diazoalkane solutions be prepared fresh and only in the quantities needed; materials that give interfering GLC peaks are formed during storage. The original hexane-diazoalkane generating solution should not be stored in ground glass-stoppered containers; however, no hazard is involved in the 5 ml test tubes containing the PCP-hexane extract plus diazoalkane. Extended exposure to air destroys the diazoalkane reagents.

The efficiency of the alkylating reaction was constant over wide concentration ranges of reactant and PCP, five-fold for the diazoalkanes and one thousand-fold for PCP. The suggested concentration of alkylating reagents and the 20 min reaction time were selected to provide the analyst with a wide safety factor while insuring consistency of results.

Methanol containing 20% water effectively removes excess diazoalkane as well as many of the interferences present due to the diazotizing reagent. No loss of PCP ether results from this treatment and the volume does not have to be adjusted, as may be necessary when diazoalkane is removed by air or nitrogen jet.

The identity of a particular derivative of PCP found by GLC is confirmed with p-values. Availability and use of several derivatives increases the confidence that a correct identification has been made. p-Values (11, 12) are especially useful for confirmation of pesticides at levels which can only be determined by electron capture gas chromatography.

The analyst can avoid changing the conditions of GLC instrumentation, which may be set up for other analyses, by selecting a derivative with a retention value compatible with existing conditions. This flexibility allows the analyst to choose a derivative which yields good chromatographic data while avoiding the time-consuming process of instrument adjustment.

Summary

The procedure described is a rapid, reproducible, and flexible method for the determination of PCP in urine, which is also applicable to the analysis of water samples. The ability to confirm the identity of PCP and to quantitate accurately in the presence of interfering compounds is facilitated by the proper selection of one or more possible derivatives.

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Determination of Trace Quantities of Anticholinesterase Pesticides

M. CRANMER[1] AND A. PEOPLES

Environmental Protection Agency, Perrine Primate Laboratory, P. O. Box 490, Perrine, Florida 33157

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A procedure has been described which is applicable to the screening of large numbers of samples for anticholinesterase activity. The method is sufficiently sensitive to detect cholinesterase depression due to as little as 30 ppt paraoxon; 3 ppb malaoxon or 2 ppm of carbaryl in either ethylene glycol or water. While the method does not allow the absolute differentiation between compounds which might be present, some classification is possible, i.e., for compounds which must be activated before they possess anticholinesterase activity.

There is currently a great emphasis on establishing the quality of our environment. A universal problem is that of air and water becoming contaminated with toxic agents. Methods for detecting these toxic agents should be reliable and if possible simple to do.

Organophosphate and carbamate insecticides are two classes of toxic chemicals which find their way into both air and water. Since these compounds are extremely toxic even in low concentrations and since absorption from air and water is efficient even trace amounts may be of biological significance. There is currently no single multiresidue method which is applicable to all organophosphate or carbamate insecticides and many of the procedures which are available lack specificity (1–3). There are several methods which are extremely sensitive for certain organophosphate insecticides that utilize gas–liquid chromatography and selective detectors. The most sensitive of these methods require 10^{-10} g of parent compound for accurate determination (4–6).

A common denominator between organophosphates and carbamates is their ability to inhibit cholinesterase. Since the toxicity of various insecticides differ markedly in their ability to inhibit cholinesterase, it is not possible to quickly determine which insecticide is present, but the detection of total anticholinesterase activity does offer the possibility of

[1] Presently: National Center for Toxicological Research, Jefferson, Arkansas 72079.

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screening air and water samples to see if there are toxic substances present that will inhibit cholinesterase.

This paper describes a method which is a modification of a sensitive procedure for the gas chromatographic determination of acetylcholinesterase (*7*). It is applicable to the screening of large numbers of samples with the confidence that if anticholinesterase components are present in a toxicologically significant form, they will be detected and their collective anticholinesterase activity determined.

METHODS

Apparatus

A MicroTek MT-220 gas chromatograph equipped with a single hydrogen flame ionization detector and a Westronics dual pen recorder were used in this study.

The incubation of the reaction was carried out with a Thermolyne Dri-Bath permanently set at 37°C. Components of the assay were added with Oxford pipettors and the reaction was carried out in a 5-ml glass-stoppered test tube.

Reagents

Reagents were obtained from the following sources: 3,3-dimethylbutyl acetate (DMBA) and 3,3-dimethylbutanol (DMB), K & K Laboratories; true cholinesterase, Type I, Sigma Chemical Company; paraoxon, Farben Fabriken-Bayer-A.G.; malaoxon, American Cyanamid; carbaryl, Union Carbide; Tween 20® emulsifier, Biscayne Chemical Laboratories. All other reagents were ACS grade.

Preparation of Reagents

A stock solution of buffer is prepared by dissolving 44.73 g potassium chloride, 4.12 g sodium barbital, and 0.55 g potassium monobasic phosphate in 20 ml water (*8*). The working solution is prepared by adding 20 ml of stock solution to 75 ml water, adjusting the pH to 8.0 with 0.1 N HCl, and diluting the solution to a final volume of 100 ml with water.

To remove butanol impurities from DMBA, five parts of DMBA are mixed with one part of acetic anhydride. The mixture is kept at 37°C for 24 hr and then washed once with water to remove the acetic anhydride and impurities. A 0.2 M solution is prepared by diluting the acetylated DMBA with working buffer and adding emulsifier to a concentration of 0.2%. The pH is then adjusted to pH 8.0 with 5% sodium hydroxide.

For gas chromatographic standards, DMB solutions containing 50, 100, 150, and 200 ng/μliter are prepared in water containing 0.1% emulsi-

fier; 2 ml of carbon disulfide (CS_2) are added to 2 ml of each standard solution, and the mixtures are shaken well and centrifuged. The top layer is discarded and an appropriate volume of the CS_2 layer is injected into the gas chromatograph. Standard curves are prepared by plotting DMB concentration vs peak height or peak area.

Concentrated formic acid (88%) is diluted 1:1 with distilled water.

A stock enzyme solution is prepared by adding 3 ml of pH 8 buffer to 50 μM units of true cholinesterase. Dilutions of 16.6 μM units/ml(A), 3.3 μM units/ml(B), 0.66 μM units/ml(C), and 0.13 μM units/ml(D) are prepared in pH 8 buffer.

Three solutions of the inhibitor of choice, i.e., carbaryl (2.0 μg/μliter, 201 ng/μliter, 20.1 ng/μliter), paraoxon (27.5 pg/μliter, 2.75 pg/μliter, 0.275 pg/μliter) and malaoxon (3.15 ng/μliter, 315 pg/μliter, 31.5 pg/μliter) are prepared in ethylene glycol and serve as standards for comparison of unknown samples.

A 0.2% bromine water solution is prepared by diluting 0.2 ml bromine with 100 ml distilled water.

Preparation of Sample

Samples of anticholinesterase compounds in water or ethylene glycol have been found to be compatible with the enzymatic system. Alcohol, ether, acetone, and other organic solvents are not acceptable, unless compared to standard curves prepared in a similar way, since they alter the activity of the enzyme solutions.

Samples of anticholinesterase compounds in water and ethylene glycol which require activation, i.e., parathion to paraoxon, must be treated with one part of a 0.2% bromine water solution to ten parts of sample solution for 1 hr at 37°C. The bromine water residue does not interfere with the acetylcholinesterase reaction.

Chromatography

Column: a glass U-tube, ¼ in × 6 ft, was packed with Johns-Manville Chromosorb 101, mesh size 80–100, and conditioned overnight at 250°C. Instrument conditions: column oven, 215°C; detector, 265°C; inlet, 245°C; air, 300 ml/min; hydrogen, 20 ml/min; nitrogen, 40 ml/min.

Procedure

Standard curves. Inhibition curves are prepared by incubating enzyme and inhibitor for 1 hr at 37°C.

To 1 ml of pH 8 buffer in a 5-ml ground glass-stoppered test tube add the amounts of Solution I (inhibitor and enzyme) designated in II and warm to 37°C. Add 0.2 ml 0.2 M DMBA and incubate at 37°C for 30

TABLE 1

Solution	Enzyme concentration			
	A	B	C	D
I μliter enzyme	100	200	500	2000
μliter inhibitor solution	10	20	50	200
II μliter of I per assay	10	50	250	1250

min. Carry a blank, containing all reagents except enzyme solution, throughout the procedure. After 30 min, stop the reaction with 0.1 ml of 44% formic acid and add 2 ml CS_2. Shake the tubes vigorously, centrifuge, and remove top aqueous layer by aspiration. Inject an appropriate volume of CS into the gas chromatography for DMB determination.

Unknown. Divide the sample to be analyzed into two parts and to 10 vol of the first part add 1 vol of 0.2% bromine water and incubate at 37°C for 1 hr. Add the nontreated and the brominated samples to the enzyme as designated in I of Table 1 and follow the same procedure.

Calculation of Inhibition

$$\% \text{ inhibition} = \frac{a - b}{a} \times 100$$

a = chart units of uninhibited sample
b = chart units of inhibited sample

Standard curves are prepared by plotting percentage of inhibition vs inhibitor concentration on semilog paper; percentage of inhibition of samples are taken from these standard curves.

Calculation of Quantity

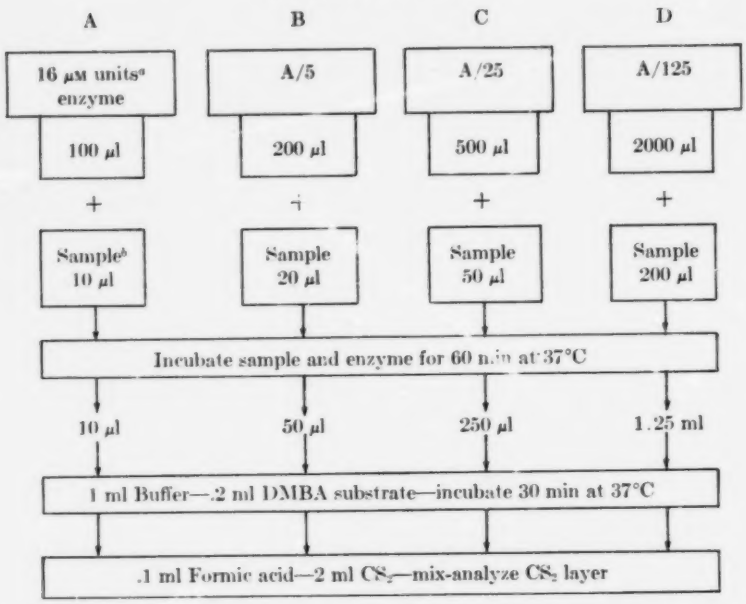
Quantification is accomplished by comparing the inhibition obtained with an unknown sample to that obtained with known concentrations of a reference inhibitor. Results would then be expressed in equivalents of paraoxon, carbaryl, malaoxon, or any inhibitor of choice.

The procedure is summarized by the flow diagram in Table 2.

RESULTS AND DISCUSSION

This assay was based on a gle method for the determination of the cholinesterase activity of small (microliters) amounts of human blood. The use of 3,3-dimethylbutyl acetate as a substrate rather than acetylcholine was determined by several preferred characteristics: (1) decreased nonenzymatic hydrolysis, (2) solubility in a solvent of low

TABLE 2
Procedure Flow Sheet



[a] A unit of activity equal to micromoles of DMBA hydrolyzed/ml solution/minute at 37°C under conditions described.

[b] The sample is divided into two parts and one part is reacted with 0.2% bromine water at 37°C for 1 hr (10 parts sample + 1 part bromine water).

hydrogen flame response, (3) increased response of 3,3-dimethyl butanol with respect to choline or acetic acid, (4) satisfactory chromatographic characteristics of both 3,3-dimethylbutyl acetate and 3,3-dimethyl butanol, and (5) equal substrate to enzyme affinity.

Figure 1 illustrates the gas chromatographic characteristics of dimethylbutyl acetate and dimethyl butanol.

It was anticipated that competitive and noncompetitive inhibitors might require different incubation periods with cholinesterase since the mechanism of inhibition is quite different. For example, the phosphorylation of the enzyme by malaoxon proceeds through two steps; (1) intact malaoxon inhibits the enzyme (competitive); (2) malaoxon is hydrolyzed by the enzyme and the enzyme is phosphorylated (slowly reversible) while carbaryl, a carbamate, acts primarily as a competitive inhibitor.

Figure 2 illustrates the inhibition of the four different dilutions of enzyme by 10 nmoles carbaryl/.1 ml enzyme solution. The reduction

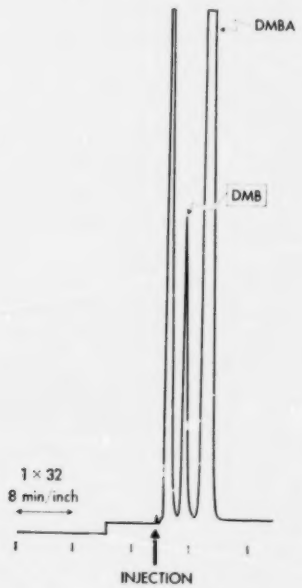


Fig. 1. Chromatogram illustrating the resolution of dimethyl butanol (DMB) and dimethylbutyl acetate using a flame ionization detector and Johns-Manville Chromosorb 101.

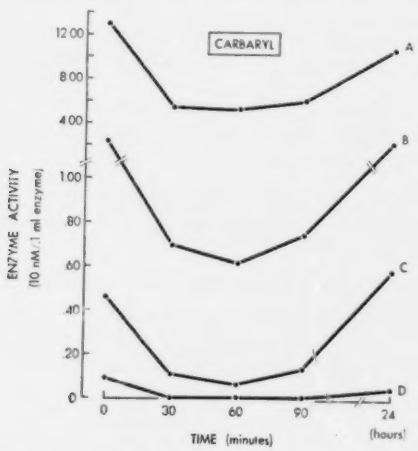


Fig. 2. Inhibition of four activities of true cholinesterase by 10 nmoles carbaryl/.1 ml enzyme vs time. Enzyme activity: (A) 16.6 μM units/ml, (B) 3.3 μM units/ml. (C) .66 μM units/ml, (D) .13 μM units/ml.

in activity approaches a maximum in 60 min. Malaoxon demonstrated the same trend as illustrated by Fig. 3. Similar results are obtained for other inhibitors including the very potent inhibitor paraoxon. These observations lead to the conclusion that a 60-min incubation period was sufficient and that there were no problems in analyzing either competitive or noncompetitive inhibitors with this assay system.

The conditions of this assay were arranged in such a way that a wide-range of concentration of cholinesterase inhibitors could be screened. Four fivefold dilutions provide a 125-fold difference in enzyme concentration available for inhibition. These dilutions are adjusted to an equivalent amount of activity present in each reaction by varying the ratios of buffer to enzyme + buffer making up the reaction mixture. For example, only 10 μliters of the most active enzyme solution (A) is added to 1 ml of buffer while 1.25 ml of the least active (D) solution is required. This compensation results in the same range of reaction product being produced for all initial enzyme concentrations (A,B,C,D) and greatly simplifies the gle determinative step. The different enzyme concentrations do not produce four identical reaction systems however, since the kinetics of the inhibition of this enzyme depend on the concentration of the enzyme and inhibitor. There is however a clear relationship between the different enzyme dilutions and the amount of inhibition which results at each inhibitor concentration.

Figure 4 illustrates the four enzyme dilutions and the inhibition of

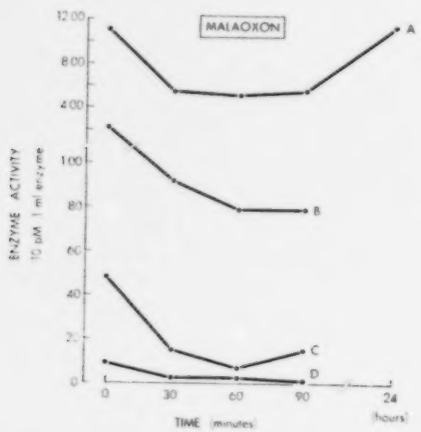


FIG. 3. Inhibition of four activities of true cholinesterase by 10 pmoles of malaoxon/.1 ml enzyme vs time. Enzyme activity: (A) 16.6 μM units/ml, (B) 3.3 μM units/ml, (C) .66 μM units/ml, (D) .13 μM units/ml.

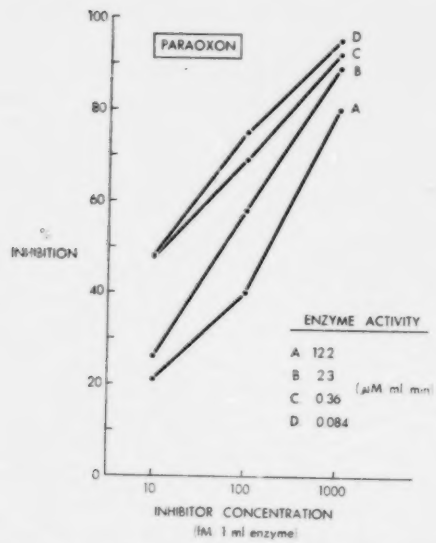


FIG. 4. % Inhibition of four true cholinesterase activities by three concentrations of paraoxon.

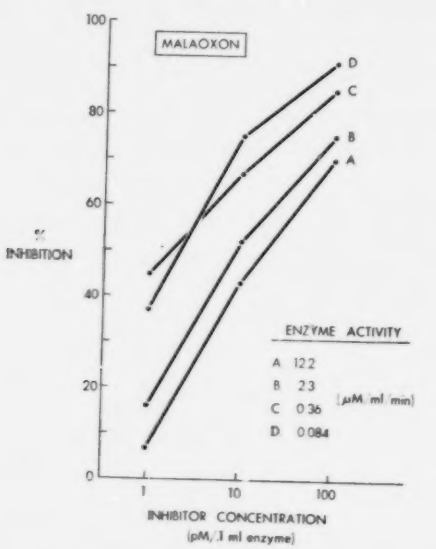


FIG. 5. % Inhibition of four true cholinesterase activities by three concentrations of malaoxon.

each dilution produced by three paraoxon concentrations differing by 100-fold. The paraoxon concentrations used are expressed in femtomoles (10^{-15}). This illustration demonstrates that for paraoxon the sensitivity of the assay is in the femtogram region.

Figure 5 illustrates the inhibition characteristics of malaoxon in the picomole range on the various enzyme dilutions. There is roughly a 100-fold difference in the inhibitory, effectiveness of paraoxon and malaoxon in this assay procedure. This difference roughly parallels the difference in the iv administered LD_{50} of the two compounds.

Figure 6 illustrates the inhibition characteristics of carbaryl in the nanomole range. There is roughly a 100,000-fold difference in the inhibitory effectiveness of paraoxon and carbaryl and again this is indicative of the order of magnitude separating the toxicities of the two compounds.

Table 3 shows the mean and standard error of six replicate samples. In each case the inhibitor concentration produced about 50% inhibition of the enzyme.

Table 3 also shows mean and standard error of day-to-day variation. The values are of seven determinations made in a 2-mo period.

The data contained in Table 4 support the observation that the assay procedure is sufficiently precise to distinguish at the 95% confidence level the difference between *1* and *3* picomoles of malaoxon.

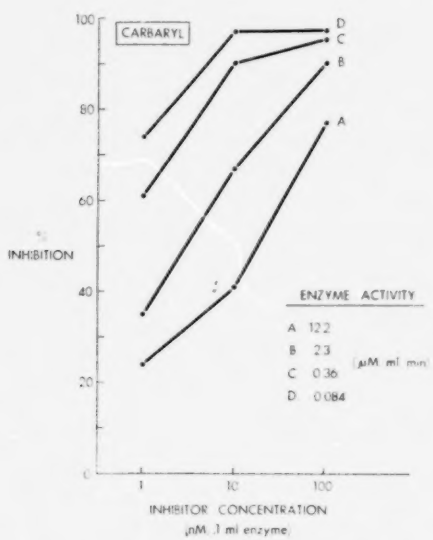


FIG. 6. % Inhibition of four true cholinesterase activities by three concentrations of carbaryl.

TABLE 3
Variability of Method
Mean and standard error for six identical samples

Carbaryl				
Enzyme activity (μM/ml/min)	A	B	C	D
Mean	5.4	.64	.065	.0046
SE	.11	.01	.001	.0004
Malaoxon				
Enzyme activity	A	B	C	D
Mean	6.1	1.0	.154	.0235
SE	.11	.02	.001	.0005
Mean and standard error of day-to-day variation of seven determinations				
Enzyme activity (μM/ml/min)	A	B	C	D
Mean	11.2	2.2	.41	.083
SE	.4	.07	.02	.003

TABLE 4
Illustration of Malaoxon Concentration and % Inhibition

Enzyme activity (μM/ml/min)	Sample (inhibitor concn in moles)	% Inhibition	
		Extrapolated[a]	Observed[b]
A	$1—1 \times 10^{-6}$	44	41
12.2	$2—3 \times 10^{-6}$	59	59
	$3—5 \times 10^{-5}$	66	66
B	$1—1 \times 10^{-6}$	51	48
2.3	$2—3 \times 10^{-6}$	64	67
	$3—5 \times 10^{-5}$	70	74
C	$1—1 \times 10^{-6}$	61	54
.36	$2—3 \times 10^{-6}$	72	73
	$3—5 \times 10^{-5}$	77	82
D	$1—1 \times 10^{-6}$	70	62
.084	$2—3 \times 10^{-6}$	81	80
	$3—5 \times 10^{-5}$	86	86

[a] Results expected by extrapolation from standard curve.
[b] Results obtained experimentally.

Samples of malathion, parathion, carbaryl, and other anticholinesterase compounds have been analyzed in both ethylene glycol, a common solvent used in air samplers and in potable water. The quantities of these compounds were determined both by standard analytical procedures and by the enzyme assay technique and produced results in good agreement.

The anticholinesterase activities of samples containing direct inhibitors and components which must be activated can be divided by determining duplicate samples of which only one is treated with bromine water.

This assay technique shows much promise for further development in the area of environmental sample evaluation.

For most screening procedures the oxidation of all samples and the use of a single, probably B, enzyme concentration would be sufficient to identify samples which might require further analytical evaluation.

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Public Health Hazards Involved in the Use of Organic Phosphorus Insecticides in Cotton Culture in the Delta Area of Mississippi[1]

GRIFFITH E. QUINBY, KENNETH C. WALKER, and WILLIAM F. DURHAM

ABSTRACT

During the summer of 1957, suspected cases of poisoning by cotton insecticides were investigated in the Mississippi Delta area. Blood cholinesterase levels of cases and of workers with maximal exposure to organic phosphorus insecticides were determined. In addition, the decay rates of insecticides and the exposure of workers were measured.

Ninety-one illnesses suspected of being poisoning due to insecticides were evaluated. Evidence was found to definitely incriminate organic phosphorus compounds as a cause of illness in two workers in formulating plants. A diagnosis of poisoning must be considered in three pilots. One fatality and one other illness were attributed to the ingestion of chlorinated hydrocarbons. In 7 of 11 crashes or disturbances of the pilot's performance about which enough information was available, three appeared to represent pilot error, one was myocardial infarction, and three—mentioned above—appeared to be mild poisoning with consequent interference with flying ability.

An epidemic characterized by fever and respiratory symptoms accounted for the majority of cases suspected of being poisoning. Epidemiologic and toxicologic investigation of this epidemic established infection and ruled out insecticides as the cause of these illnesses.

Groups of persons selected because of their exposures to methyl parathion or to Guthion resulting from their occupation or from their residing in treated areas were studied for evidence of poisoning. No clinical evidence of poisoning or significant cholinesterase depletion was found.

The half-life for methyl parathion on cotton leaves was found to be less than 1 hour. Exposure studies on checkers corroborated this rapid decay rate. Most of the residue exposure was on the hands.

The half-life of Guthion spray and dust on cotton leaves appeared to be about 2 to 4 days. Exposure studies indicated that most of the residue exposure was to the hands.

The reported failure of chlorinated hydrocarbon insecticides to achieve satisfactory control of the boll weevil in the Mississippi Delta in 1955 led to the increased use of

[1] From the Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, Wenatchee, Washington. This paper was endorsed and communicated for publication by K. D. Quarterman. Accepted for publication June 13, 1958.

Table 1.—Application rate, concentration, and length of cycle of organic phosphorus insecticides as used in the Delta area of Mississippi in 1957.

Insecticide	Application Rate (Lb./Acre)	Concentration (Per Cent)					Length of Cycle (Days)
		Spray			Dust		
		Emulsifiable Concentrate	Dilute				
			Ground	Airplane	Ground	Airplane	
Malathion	0.5 –1.0	65	1 –2	6–12	4 –10	4 –10	3–5
Methyl parathion	0.25–0.50	25	0.5–1.5	3–6	2.5– 5.0	2.5– 5.0	3–5
Guthion	0.25	20	0.5	3	—	—	5–7

organic phosphorus compounds, especially malathion [*O,O*-dimethyl S-(1,2-dicarbethoxyethyl)dithiophosphate] and methyl parathion (*O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate) (Furr & Lloyd 1957). An even greater use of methyl parathion and Guthion [S-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-ylmethyl) *O,O*-dimethyl phosphorodithioate] was anticipated for 1957. It was believed by some in Mississippi that poisoning had occurred and was occurring in both occupationally exposed persons and in residents of the area where these insecticides were being used. The idea also prevailed that the poisoning of pilots was responsible for an alleged increase in the airplane accident rate.

Various state and federal agricultural agencies in the Mississippi Delta area requested the Communicable Disease Center of the Public Health Service to investigate the hazards associated with the use of these insecticides. This report presents the results of the study.

The introduction of new or more toxic insecticides into an area has often led to fears for the safety of the populace. Sumerford *et al.* (1953) in 1951 studied cholinesterase response and symptomatology from exposure to organic phosphorus insecticides in the fruit orchards of North Central Washington. The survey was continued in 1952 and 1953 (Hayes *et al.* 1957). There was, at the beginning, considerable anxiety in the community because of deaths and illnesses attributed to minimal exposure to these compounds. It was found that persons definitely exposed to parathion showed a significant reduction in average cholinesterase values and an increase in illnesses consistent with poisoning during the period of their exposure. No evidence was found of either reduced cholinesterase activity or increased illness during the spray season in persons with little exposure to insecticides, including those who merely lived within the treated area.

Under somewhat similar conditions, Fowler (1953) investigated in 1952 the health hazards involved in the use of the newer economic poisons in the control of cotton insects in the Mississippi Delta. At that time the chlorinated hydrocarbon compounds were of most concern. Although he reported seven cases of acute insecticide poisoning following extensive exposure, Fowler found no evidence of any chronic intoxication by insecticides and no indication that they acted as a contributing cause in diseases generally recognized as having other etiologies.

Quinby & Lemmon (1958) have recently described outbreaks of poisonings in crop workers from dermal exposure to parathion residues on foliage and fruit. Agricultural authorities in Mississippi were familiar with these findings and were, therefore, fully aware that poisoning by at least one organic phosphorus insecticide was possible through exposure to residues as well as through contamination during application.

Conditions of Study.—The problem in the Delta area was approached in four ways: (1) Suspected cases of poisoning were investigated. (2) Blood cholinesterase levels of cases and of workers with maximal exposure to organic phosphorus insecticides were determined. (3) The decay rates of insecticide residues on cotton were followed. (4) The respiratory and dermal exposure of cotton checkers was measured.

The studies were carried out during the summer of

Table 2.—Evaluation of reported cases suspected of being poisoning by cotton insecticides, Mississippi Delta area, 1954–57.

Evaluation[a]	Organic Phosphorus Insecticides					Chlorinated Hydrocarbons	Unspecified Insecticides	Total
	Parathion	Methyl Parathion	Guthion	Malathion	Unspecified			
Fatal Poisoning	0	0	0	0	0	1 (Endrin)	0	1
Non-fatal Poisoning:								
Cholinesterase confirmed	0	0	0	0	0	—	—	0
Clinically consistent only	2	1	1	1	0	1	0	6
Asymptomatic cholinesterase depletion	0	3	0	0	0	—	—	3
Doubtful poisoning	1	2	0	0	0	0	0	3
Exposed, illness unrelated	0	21[b]	1	13	2	0	4	41
Unexposed, illness unrelated	1	28[b]	0	0	6	0	2	37
Insufficient data	0	2	0	0	2	2	3	9
Total	4	57	2	14	10	4	9	100

[a] Tabulated in order of relative certainty of poisoning.
[b] Epidemic respiratory diseases.

1957 from headquarters at the Delta Branch Experiment Station, Stoneville, Mississippi. In addition to intensive investigations carried out at the Station and at selected places nearby, reports were solicited from 13 counties in West Central Mississippi which included an area limited approximately by Greenville, Clarksdale, Greenwood, and Vicksburg.

All practicing physicians in these 13 Delta counties were advised of the study by letter and requested to report all cases of suspected poisoning; laboratory tests and other assistance were offered in connection with these cases. A technical review of the toxicology of the organic phosphorus insecticides with emphasis on early recognition, laboratory confirmation, and treatment was presented before the staff of each of 10 hospitals in the area. During the series of visits to each hospital, their records since 1954 were searched for past and current cases of poisoning. The health departments of the nearby states were requested to report any cases of insecticide poisoning and were alerted to watch especially for residue contact poisoning in agriculture workers. A few individual cases were reported from these states and are included in the tabulations.

As a result of these activities, a total of 100 cases of suspected insecticide poisoning were studied, including 11 episodes involving either an airplane crash or a disturbance of a pilot's performance.

During the course of the clinical investigations and planned blood cholinesterase surveys, the cholinesterase activity values of about 800 blood samples were generously made available by private laboratories. There were multiple samples on some persons. In addition, 131 blood samples including all those shown later in table 3 were analyzed at the Technical Development Laboratories, Savannah, Georgia. All cholinesterase determinations were carried out by the Michel procedure (Michel 1949) unless specified otherwise.

Ninety-two persons representing eight occupational groups with varying levels of exposure to methyl parathion were studied to determine the effect of this exposure on their health. Exposure and clinical histories were obtained from all 92 persons, cholinesterase activity levels were determined. An effort was made to include within each group those persons with the heaviest exposure. In a similar manner, 32 persons were studied in a survey on Guthion. Since malathion has a very low mammalian toxicity, no special exposure studies were made on this compound but, of course, alleged poisonings were investigated.

In preparation for studies of the decay rates of insecticide residues and also for measurements of the exposure of cotton checkers, insecticide applications were made by the staff of the Delta Branch Experiment Station, Stoneville, Miss., using ground equipment. A machine similar to commercial ones but especially designed for precise application was used. For each compound, residue decay determinations and exposure studies were carried out concurrently in the same plot. During this work, air temperatures taken near the leaves ranged from 59 to 97° F. and the relative humidity was high.

After the spray had dried (less than 5 minutes) or the dust had settled, leaves were removed and placed in a new paper bag. From cotton in the various rows, leaves were removed from representative positions on the top and middle canopy of the plants and some from the interior, but none from the lower third near the ground.

The time interval between the removal of the leaves from the plant and their extraction was held to a minimum. The decay time represents the interval between application and extraction. All references to leaf residue are expressed in terms of square inches of leaf, not leaf surfaces. Most of the samples were prepared by cutting 1 sq. in. from each of 50 leaves stacked together. The cuttings were immediately placed in a reagent bottle and washed with three serial rinsings of approximately 100 ml. of ethyl alcohol. The bottle was shaken vigorously 20 times in about 30 seconds for each rinsing. The alcohol extract was mailed to the laboratory 2 to 3 days away. Recovery from control samples of methyl parathion shipped in the

Table 3.—Mean blood cholinesterase activities of occupational groups arranged in estimated order of decreasing exposure to methyl parathion or to Guthion.

Occupational Group	Health Status[a]	No. of Persons	Average Cholinesterase Activity (ΔpH/Hr.)	
			R.B.C.	Plasma
Exposure to methyl parathion				
Loaders	Well	6	0.62	0.84
Applicators	Sick	4	.77	.85
	Well	1	.89	1.20
Warehousemen	Sick	1	.65	1.16
Pilots	Sick	2	.83	.83
	Well	4	.62	1.07
Experimental entomologists	Well	4	.71	.69
Checkers	Well	21	.78	.92
Choppers or hoe-hands	Sick	3	.63	.38
	Well	10	.72	.90
Residents	Sick	26	.72	.86
	Well	10	.72	.97
Total or mean	Sick	36	.72	.83
	Well	56	.73	.92
Exposure to Guthion				
Experimental entomologist	Well	1	0.75	0.58
Flagmen	Well	3	.76	.88
Pilot	Sick	1	.88	.82
Irrigators	Well	11	.77	.77
Checkers	Well	4	.73	.94
Field supervisors	Well	4	.83	1.08
Residents	Well	8	.79	.96
Total or mean	Sick	1	.88	.82
	Well	31	.78	.88

[a] Respiratory diseases were epidemic.

same way indicated no significant decay en route.

Methyl parathion was determined by the method of Averell & Norris (1948) which produces a magenta color. Some 2,-3,-and 4-day-old residues gave no leaves gave varying shades of brownish-magenta on final color development. The brown component probably affected the final optical density and tended to give values slightly higher than true values. These values are interpreted as of questionable accuracy. In general, these values were not more than 50 micrograms per total sample. Samples collected 5 or more days after spraying gave no detectable magenta on final color development and the values calculated from the optical densities were below the limit of sensitivity of the method (20 micrograms). A blank value of 13 micrograms was used to correct all analyses of leaves.

Exposure studies were carried out using techniques similar to those described by Batchelor & Walker (1954) and by Batchelor *et al.* (1956). Recovery studies were carried out to estimate the possible loss of sample from exposed garments during the mailing time of 2 to 3 days. Similar garments were treated at the laboratory with known amounts of methyl parathion, saturated with saline to simulate perspiration, and stored under even hotter conditions than those to which air mail would subject them. Losses were insignificant from the dry specimens but as high as 50% from wet ones.

Guthion was determined by a modification of an unpublished method furnished by the Chemagro Corporation.

Spray practices concerned with the control of cotton insects in the Mississippi Delta during 1957 may be summarized as follows:

The proportion of cotton growers in the Delta area using organic phosphorus insecticides varied from less than a third at the beginning of the 1957 season to over half at the end. Early, malathion was by far the most commonly used organic phosphorous compound, but by the end of the season the use of methyl parathion had increased until it probably exceeded malathion, at least in acreage covered. A smaller amount of Guthion was used. Parathion (*O,O*-diethyl-*O*-*p*-nitrophenyl thiophosphate), demeton [*O,O*-diethyl S-(2-ethylthio) ethyl phosphorothioate mixture with *O,O*-diethyl *O*-(2-ethylthio) ethyl phosphorothioate], and other organic phosphorus insecticides were rarely employed.

The ecology of the boll weevil is such that control has been obtained by spraying or dusting the canopy of the cotton fields. Sprays have been more popular than dusts. The application rate, concentration, and length of cycle for organic phosphorus insecticides used in the Delta area of Mississippi are shown in table 1. The volume rate of mixed spray was about 5 to 6 gallons per acre by ground equipment and about 1 gallon per acre by airplane. Control of various combinations of cotton pests frequently required that mixtures of insecticides be applied at these same low volumes per acre. Some applications of larger than recommended amounts of insecticide per acre were made. Both of these practices sometimes resulted in an increased concentration of petroleum distillate vehicle in the finished spray. Dust applications ranged from 5 to 20 pounds of diluted material per acre.

Early in the 1957 spray season, two statements on safety precautions had been printed by the Delta Council and the Mississippi Aeronautics Commission. A series of newspaper articles on safety had been sponsored by the Agricultural Extension Service. A supplementary statement on first-aid was issued later in the newspapers by the State Board of Health and the State Medical Association. In spite of these warnings, violations of recommended safety precautions were common. Often there was a practical reason for these violations, but some were readily preventable.

The more important of these safety violations were as follows:

1. Washing, bathing, and laundering facilities were generally lacking or inadequate.
2. Many pilots loaded their own planes.
3. Pilots usually did not cut off their motors during reloading.
4. Opened and "empty" spray containers were left accessible to children and other uninformed persons.
5. Discarded 5-gallon concentrate containers and dust sacks were used as permanent swath-markers or flags.
6. Loading and storage facilities for insecticides were unsafe.
7. Hoses connected to drinking water supply were placed into the spray mix.
8. The real toxic hazards of insecticides were concealed or minimized by some growers and sales agents.
9. There was a failure to provide for the blood cholinesterase testing of some pilots, loaders, and other handlers when they were recognized to have had spillage accidents or other excessive exposure.

It was observed that, in the course of these violations and in accidents, many workers tolerated heavy exposures to the more commonly used insecticides, malathion and methyl parathion, without developing symptoms.

RESULTS.—*Clinical Investigations.*—Of the 100 suspected cases of poisoning on which some data were available, enough was learned about 91 cases to evaluate them. These cases are tabulated in table 2 in order of their relative certainty of poisoning and on the basis of compound originally suspected.

No fatal case of organic phosphorus poisoning was found during the period covered by the study (1954–57). A child did die, apparently from ingesting endrin. The evidence indicates the child swallowed enough endrin from a can to cause convulsions and death.

Two nonfatal cases clinically consistent with parathion poisoning occurred in 1954 and 1955 in formulating plants. Cholinesterase tests were not made in these cases. In this connection, it should be noted that the volume of parathion formulated and used was small in former years and almost nil in 1957. By contrast, the amount of methyl parathion formulated and applied in the Delta area during 1956 and 1957 was considerable.

Three cases consistent with mild organic phosphorus insecticide poisoning sufficient to interfere with the efficiency of pilots are described below in detail; they were due to methyl parathion, Guthion, and malathion, respectively. One case consistent with poisoning from ingesting a mixture of BHC and DDT was also reported.

Asymptomatic cholinesterase depletion was found in three loaders. Two of them who had violated safety instructions had red blood cell cholinesterase activity levels below the usual symptomatic level for persons with small but repeated exposure. The third loader's post-exposure level was in the low normal range but was significantly below his pre-exposure values.

Three cases (1 parathion and 2 methyl parathion) were classified as doubtful. Their clinical pictures and exposure histories were not consistent with poisoning, but tests had not been done to rule out poisoning with certainty.

There were 78 persons each of whose illness was suspected of being poisoning but was considered after study to have some other cause. The histories indicated that 41 of these persons were workers in cotton fields or had been exposed in some other way. The remaining 37 were essentially unexposed. Many of these cases were ill in epidemics during the summer of 1957. Only one physician attributed any substantial number of these illnesses to poisoning. However, many people were concerned over the claims of this one physician that the epidemics were due to poisoning, especially when widespread newspaper and radio publicity was given to this possibility. Poisoning was reasonably ruled out by case histories and blood cholinesterase tests in one epidemic. In addition to the toxicologic studies in this particular area, the Mississippi State Board of Health sent an epidemiologic team to collect specimens and symptom data. Their findings gave direct support to the conclusion that this epidemic was caused by respiratory infections. Less intensive toxicologic surveys were conducted in two other smaller population groups suffering similar waves of respiratory disease. Neither of these surveys showed any indication of poisoning.

Several other reports not connected with the epidemic involved mistakes in initial diagnosis. It is not surprising that the public became alarmed in view of the extent to which stories based on rumors and on misdiagnosis were bandied around in the newspapers, by radio, and by word of mouth. These misconceptions reached their acme when the confusion between poisoning and the epidemic respiratory disease caused the etiologic agent of the epidemic to be called the "cotton poisoning virus."

Cases involving pilots are of special interest because pilots may be exposed to considerable contamination and because minor poisoning of a pilot might have far more serious consequences then similar poisoning of a person whose life did not depend on the skillful manipulation of an intricate machine. Eleven instances of airplane crashes or disturbances of a pilot's behavior were investigated. One occurred in 1955, three in 1956, and seven in 1957. Unfortunately, so little information was obtained in four of the cases that no decision whatever can be made regarding their probable causes. In two cases, it is not even established that the pilots were exposed to organic phosphorus insecticides. Three cases appeared to be clear examples of pilot error with the possibility of poisoning ruled out by adequate information. One case was myocardial infarction.

A diagnosis of poisoning must be considered in three of the cases which involved significant exposure and incoordination or other symptoms. The fact that blood cholinesterase values were normal shortly after the accidents offers no support to this view, but it in no way rules out local effects (Upholt *et al.* 1956) or even mild systemic poisoning (Sumerford *et al.* 1953). Obviously, mild intoxication or the visual disturbances caused by the local (or systemic) action of certain organic phosphorus insecticides would be sufficient to interfere with a pilot's efficiency. The three cases were:

R.W.: This pilot crashed in 1956 while carrying malathion dust, but he had reportedly been "affected" by methyl parathion some weeks previously. While his many fractures and other severe injuries adequately explained his death, they did not explain the erratic flying which a witness observed preceding the crash. A post-mortem Michel blood cholinesterase test was reported as normal (RBC, 81%; plasma 79%); however, the effect of earlier transfusions on this result cannot be stated.

F.R.: This 42-year-old pilot did not spray his load of methyl parathion and had difficulty landing on August 12, 1957 because he was dizzy, unable to think clearly, and had double vision. He vomited while still in the air. There was no exposure to any other organic phosphorus insecticide. The pilot frequently had seen spillage into his cockpit and specifically recalled gross exposure 1 and 5 days earlier. He had worked long hours and eaten at irregular intervals. A screening blood cholinesterase test by a hospital laboratory showed about 66% of normal on July 31 and about 42% of normal on August 12, the day of his landing difficulty. However, by the Michel procedure, his level was normal on this date (RBC, 0.79; plasma, 0.83 ΔpH/hr.). The skin flushed following a single dose of atropine which argues against severe systemic poisoning. At the time of physical examination, no pupillary changes were noted. However, the most interesting feature of the case was that the pilot found that his visual disturbance was corrected when he shut one eye. In this way, he was able to see clearly and land his plane safely. This reaction strongly suggests the imbalance of vision which can be caused by the local action of organic phosphorus insecticides (Upholt *et al.* 1956).

J.M.: This pilot, on August 20, 1957, had spilled Guthion concentrate on his hands but washed it off within 3 minutes. During the previous 10 days, he had been loading methyl parathion and had been contaminated to the point that the white dust could be seen on his skin. Following the spillage on August 20, he had visual disturbances, headache, tightness in the chest, abdominal cramps, nausea, vomiting and weakness, and some excess salivation while flying. He had difficulty in landing but did not crash. After landing, he was improved by a dose of atropine but still showed signs of hyperperistalsis on physical examination. All of these circumstances pointed to poisoning. He had had a sore throat for 5 days, but respiratory disease was epidemic in the area at the time. However, there was no evidence that the symptoms listed above were related to respiratory disease. The fact that the pilot never developed muscle fasciculations or pin-point pupils and was found to have a normal blood cholinesterase level (RBC, 0.88; plasma, 0.82 ΔpH/hr.) argued against severe systemic poisoning.

Blood Cholinesterase Survey.—The respiratory epidemics afforded an excellent opportunity to compare occupationally exposed groups and residents, both sick and well, in areas of heavy and frequent application of methyl parathion. The staff of the Delta Branch Experiment Station furnished representatives of the several groups (including loaders, applicators, pilots, research entomologists, and many checkers). Additional subjects in these occupational groups as well as choppers and one warehouseman were obtained from cotton plantations not associated with the agricultural tests of the station. An additional group of the most heavily exposed commercial checkers were tested. These checkers walked through cotton sometimes from dawn to dark without certain knowledge of the age or nature of the residues. Their clothing became soaked from the dew-wet, residue-laden leaves. At times they crawled on their hands and knees to search for weevils. Although nearly all these

commercial checkers had occasional exposures to Guthion residues and frequent exposures to malathion, they were classified as primarily exposed to methyl parathion on the basis that it was their most hazardous skin exposure. They had been concerned enough about their risk to have had pre-exposure blood tests done at their own expense.

The results of the blood tests of these occupational groups are shown in table 3. All of the 92 persons had values within the range of normal except for two loaders and one chopper. The two loaders had violated safety precautions; they were previously referred to under the section on clinical investigations. The one chopper with a definitely depleted plasma cholinesterase level was a hypertensive pregnant woman ill with respiratory disease. Her red cell cholinesterase activity was normal. She may have suffered liver damage from several toxemias of pregnancies.

It may be seen from table 3 that there was no appreciable difference in average cholinesterase level between the sick and the well persons within each occupational group, or between the sick and the well persons for the entire population sampled.

Only one plantation was found where the application of Guthion was sufficient to warrant conducting a blood survey. The blood testing was done on the sixth day of the fifth cycle on a 7-day aerial-spray schedule. A Guthion residue of 1.4 micrograms/sq. in. of cotton leaf was found at this time. Because of their substantial exposure to each compound, three individuals (a pilot, a checker, and a research entomologist) from the methyl parathion study were also included in the Guthion study. The residents lived in houses which were surrounded on three sides by cotton. Several choppers or irrigators also lived in houses in the treated cotton fields.

As can be seen in table 3, the mean cholinesterase values for each occupational group were within the range of normal, as were all individuals who made up the group.

Hundreds of other individuals from the same occupational groups were being tested by three private laboratories during and after the surveys summarized in table 3. As results from the private laboratories were made available, the 12 persons having levels below 0.50 ΔpH/hr. were studied in more detail with regard to clinical and exposure history. These low values occurred in less than 2% of the persons tested and the clinical and exposure histories ruled out symptomatic poisoning from organic phosphorus insecticides in all 12.

Residue and Exposure Studies.—The decay curves for two trials with methyl parathion residues are shown in figure 1. There is good agreement between values in the two trials. It appears from these curves that, under the conditions studied, the decay of methyl parathion on cotton leaves was quite rapid. The half-life appears to be less than 1 hour.

This rapid decay rate appears to be in reasonable conformity with previously reported rate of loss of residues of methyl parathion. Thus, Fahey *et al.* (1952) compared the longevity of parathion, methyl parathion, and EPN (*O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate) on apple foliage in 1952. They found that the 24-hour residues of methyl parathion were 30% of the 1-hour baseline residue.

One possible explanation of the rapid decay rate noted

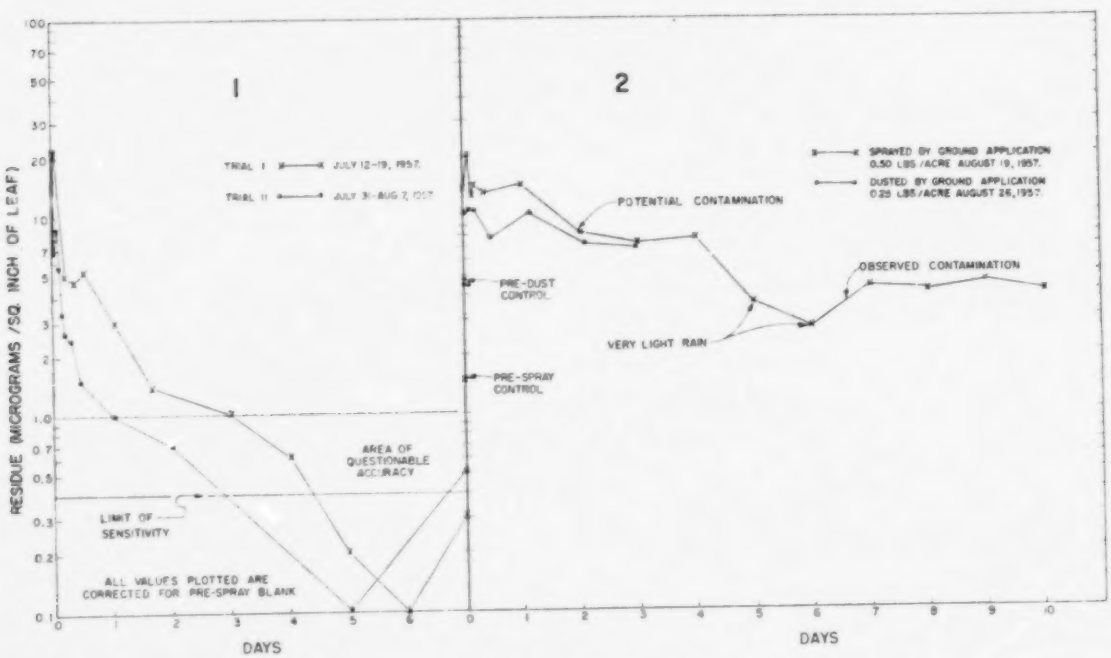

FIG. 1.—Decay rates of methyl parathion sprayed by ground application on cotton at 0.5 lb./acre (59 to 97° F.).
FIG. 2.—Decay rates of Guthion (59 to 97° F.).

Table 4.—Exposure of various body parts of checkers to recent ground or airplane application of methyl parathion spray to cotton.

	Ground (0.5 Lb./Acre)						Airplane (0.37 Lb./Acre)	
	Age of Residue						Aged 24–25 Hours	
	5–65 Minutes		1.5–3.5 Hours		4–5 Hours			
Test Garments	Micrograms per Hour[a]	Per Cent of Total	Micrograms per Hour[b]	Per Cent of Total	Micrograms per Hour[a]	Per Cent of Total	Micrograms per Hour[a]	Per Cent of Total
Respirator Pads	<20	—	<10[c]	—	<20	—	No sample	—
Gloves	2693	84	2218	87	557	81	435	60
Sleeves	417	13	288	11	75	11	205	28
T-Shirt	97	3	56	2	55	8	90	12
Total	3207	100	2562	100	687	100	730	100

[a] Based on a total of 14 garments on two men.
[b] Based on a total of seven garments on one man; exposure time, 2 hours.
[c] Experimental limit of analytical procedure was 20 micrograms when the sampling period was 1 hour and 10 micrograms when the sampling period was 2 hours.

for methyl parathion is the fact that, as described by Gunther & Blinn (1955), respiration of a leaf provides excellent opportunity for the co-distillation of steam-volatile insecticides, including those with comparatively low vapor pressures. In this connection, the high concentration of aromatic petroleum distillate in the spray formulations used in the Delta may be of importance.

This rapid decay rate of methyl parathion residues as determined by chemical studies agrees well with entomological observations. Furr & Lloyd (1957) reported that methyl parathion applied at the rate of 0.25 pound per acre on cotton lost its insecticidal effectiveness for boll weevils within 6 hours.

The results of the study of exposure of checkers to methyl parathion are shown in table 4. Right and left garments were both included. The gloves accounted for the majority of the residue recovered. The cotton sleeves and the T-shirts picked up lesser amounts. No detectable quantity was found on the respirator pads. The relative constancy of the per cent of total recovery for each test garment irrespective of the age of the residue was striking.

The total weight of methyl parathion residue obtained by all the test garments of the average checker was about 3 milligrams per hour in the first hour after spraying and less than 1 milligram per hour 4 hours after spraying. It is felt that the checker's opening of the squares with his fingers probably accounts for the majority of his exposure.

It was of interest to compare the decrease of residues on the test garments with the decay curves. The residues deposited on the test garments during the fifth hour were 79% less than during the first hour, comparing closely with the 78 and 88% decay of leaf residues in trials I and II for comparable times. A similar correlation was observed between exposure and residue decay for 4 and 24 hours.

Blood cholinesterase tests on these checkers performed 24 hours after the measured exposures showed no significant change from pre-exposure levels.

The rapid decay of methyl parathion and its consequent low residual toxicity were further supported by two observations. One grower allowed his children and calves free access to a cotton field which was treated repeatedly with 1.5% methyl parathion dust. Neither children nor livestock showed any ill effects from this exposure. The second incident involved 35 head of cattle which escaped from fencing and during the night ate the tops out of about 2 acres of cotton covered with the residue from five methyl parathion applications. The freshest residue was only 36 to 48 hours old. None of these cattle showed any harmful effects.

The residue decay curves for Guthion are shown in

Table 5.—Exposure of various body parts of a checker to recent application of guthion spray or dust to cotton.

	Spray (0.5 Lb./Acre)				Dust (0.25 Lb./Acre)			
	Age of Residue							
	5–35 Minutes		4–4.5 Hours		0–60 Minutes on Dew-Wet Leaves		4–5 Hours on Dry Leaves	
Test Garments	Micrograms per Hour[a]	Per Cent of Total	Micrograms per Hour[a]	Per Cent of Total	Micrograms per Hour	Per Cent of Total	Micrograms per Hour	Per Cent of Total
Respirator Pads	<40[b]	—	<40	—	<20	—	23	—
Gloves	5632	73	3910	74	10,720	58	>4072[c]	70
Sleeves	1584	20	946	18	4,450	24	717	12
T-Shirt	520	7	418	8	3,213	18	>1043[d]	18
Total	7756	100	5274	100	18,383	100	>5855	100

[a] Based on 30-minute exposure.
[b] Experimental limit of analytical procedure was 20 micrograms.
[c] Visible loss of dust from one glove during removal.
[d] Part of sample was lost during analysis.

figure 2. Although the application rate for the spray was twice that for the dust, previous treatment of the dust field had left a sufficient residue (about 5 micrograms/sq. in.) so that the application studied built up a total residue approximately equal to that from spraying. The decay rate of Guthion on cotton leaves was relatively slow. Its half-life was about 2 to 4 days.

In the test with the spray, traces of rain on the fifth and sixth days appeared to remove part of the residue. The slight rise in spray residue for the seventh day was associated with observed drift contamination from adjacent plots. Entomological experience with Guthion also paralleled chemical decay studies (Furr & Lloyd 1957).

One spot check in another field sprayed by airplane with 0.25 pound Guthion per acre showed 1.4 micrograms/sq.in. of leaf on the sixth day of a 7-day cycle.

The results of the exposure studies with Guthion are shown in table 5. The gloves accounted for the majority of the contamination. The sleeves and T-shirts picked up lesser amounts. A detectable amount was found on only one respirator pad. As was true for methyl parathion, the per cent of total recovery for each test garment was relatively constant regardless of the age of the residue.

For Guthion spray, the exposure represented by all the test garments was calculated to be about 8 mg. per hour in the first hour after spraying and about 5 mg. per hour in the fifth hour. The checkers' test garments picked up from the fresh dew-wet residues about 18 mg. of Guthion in the first hour. Four hours later, when the leaves had dried, their test garments retained only about 6 mg. per hour. No effect of this exposure on blood cholinesterase levels was found.

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Edited by

E.BOYLAND

D.Sc.

Professor of Biochemistry

Chester Beatty Research Institute, Institute of Cancer Research:
Royal Cancer Hospital, London,

and

R. GOULDINE

B.Sc., M.D., M.R.C.P.

Director, Poisons Reference Service, Guy's Hospital, London

Published by

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CHAPTER 10

TESTS IN MAN

WAYLAND J. HAYES, JNR.

INTRODUCTION

Limitations of Animal Studies

The basic reason for making studies in man rather than limiting them to animals is that species are not identical. To be sure, useful laboratory models may be found for many of the problems that require study. A number of human diseases may be reproduced, or at least approximated, in animals. Many toxic effects also may be produced in the laboratory. Two difficulties remain, however: (1) Some diseases and conditions have never been produced in animals. (2) Even for conditions that can be caused in animals, the degree of correspondence may be imperfect. For example, rheumatic fever and various forms of systemic hypersensitivity to drugs never have been produced satisfactorily in animals, and drugs to combat these conditions must be tested in man. The paralytic effects of tri-orthocresyl phosphate in man were faithfully reproduced in chickens (Smith and Lillie, 1931). The same was true of the paralytic effects of mipafox (Barnes and Denz, 1953). However, some other organic phosphorous compounds, even though they produce a similar muscular weakness in chickens under experimental conditions (Durham, Gaines and Hayes, 1956), have produced no neurotoxicity in man in spite of extensive commercial use.

In some instances species differences in the response to therapeutic or toxic agents have been explained by differences in the biotransformation of the compound in the different forms. This variation in the biotransformation may be qualitative (Williams, 1963), quantitative (Brodie, 1964), or both. The recognized variation between two species is like the visible part of an iceberg. The unknown, and often unsuspected variation, corresponds to the submerged portion.

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Certain Tests in Man Constitute Established Medical Practice

The most important advance that has been made in our times regarding planned tests in man is the recognition that they constitute established medical practice, at least for evaluating the effectiveness of drugs. Testing, as such, is not new. It has been carried on in one degree or another since organized medicine came into existence. Occasional systematic tests were carried on at least as early as 1835 when Dr. P. G. A. Louis investigated the effect of bleeding on the survival of patients with pneumonia. He found that the longer bleeding was delayed after the onset of illness, the higher the proportion of patients who survived. He concluded quite rightly that if bleeding had not been carried out at all, the results would have been even better. Most people in the medical profession are familiar with the fact that tests on the efficacy of drugs are routine. It is also generally known among the profession that such studies are being carried out with improved design and increasing skill. If the frequency of these tests is not sufficient evidence that they constitute established medical practice, then certainly this status was achieved in October, 1962, when the 'Drug Amendments Act of 1962' which amends the Federal Food, Drug and Cosmetic Act (21 U.S.C. 321 *et. seq*) was passed in the United States of America requiring that the effectiveness of all new drugs must be tested in man before the drug can be licensed for sale.

Strangely enough, the law which requires that the effectiveness of drugs be tested in man does not require specifically that their safety be tested in this way. It does demand that safety be tested in animals and that any untoward effects in man be reported promptly. If injury of sufficient importance is demonstrated, licence for sale of the drug will be withheld or withdrawn, depending on whether or not the drug has actually reached the market. The fact that testing of effectiveness in man is required directly but testing of safety in man is required only indirectly could be a matter of semantics, but the difference probably reflects a certain general ambivalence in present thinking about tests in man. This ambivalence may be explained on the one hand by an ever present awareness that such tests are needed and on the other hand by a combination of fear they will be misused and ignorance of the manner in which these tests are carried out under proper scientific supervision.

The author believes that much of the ambivalence in present thinking about tests in man can be attributed to the fact that one of the basic ethical considerations on which such tests depend has hardly been mentioned, much less discussed. The basic question is

whether it is right to introduce a biologically active chemical commercially without first submitting it to careful observation in a limited number of people exposed to it under conditions as accurately controlled as possible. The only alternative is to introduce the chemical directly from the laboratory to the public under conditions that necessarily minimize control and discourage observation. If it is decided that the ethical thing is to test chemicals purposefully and scientifically in man rather than leaving the matter to accident, then, and only then, is a second ethical question raised, namely: How are the tests to be carried out? The first question is whether to test in man, not whether to test in volunteers. Many valuable scientific studies can be made in workers, although volunteers present real advantages in some situations.

This paper is based on the proposition that biologically active substances that may eventually be released to the public ought to be tested in man. The fact that man is unique is taken as axiomatic. The remainder of the paper is devoted to the ethical, legal, and practical considerations involved in testing.

Kinds of Study

A number of authors have recognized tacitly (Medical Research Council, 1964) or expressly (Académie Nationale de Médecine, 1952; Hayes, 1965) that there are different kinds of study in man. Although different terms may be used, all authors seem agreed that the basic distinction is between: (*a*) studies of diagnostic procedures or therapy, and (*b*) studies of physiology in the broadest sense.

Subjects for the first kind of study are usually sick at the time of the study. They freely consent* to a calculated risk in undertaking the study, but they do so in the hope that the diagnostic procedure or treatment will be of benefit to their own health. They usually receive no other reward. Such studies are generally carried out according to a sophisticated design, frequently by the double blind technique. However, it is generally recognized that if the disease being diagnosed or treated is sufficiently rare, such studies must be carried out with a less formal plan, although with no less skill. For example, a promising new treatment for an unusual form of poisoning must be used when the opportunity arises or it will not be used at all.

* In the case of patients who are unable to give consent because of their age or medical condition, consent should be obtained from the parents or next of kin, just as is true in connexion with any diagnostic procedure or therapy for such patients. It is agreed (Freund, 1965) that parents or guardians may consent for a child (or other legal incompetent) in connexion with acts for the welfare or benefit of the child (or other legal incompetent).

Subjects for the second kind of study may be sick or well, but in either event the state of their health is not in itself critical to the study, and the subjects have no reason to suppose they will be healthier because of the test. They are often paid or given some other benefit in exchange for their service and the calculated risk to which they freely consent.* Here again, the formality of the plan of study has to depend on the situation. For example, preselected groups of men—including one or more control groups—have been studied in regard to critical ambient concentrations of oxygen and carbon dioxide (Consolazio and his colleagues, 1947), and the safety of the insecticide malathion (Golz, 1959; Hayes and his colleagues, 1960). On the other hand, Beaumont (1833) had no possibility of using controls or of checking his observations in a number of preselected subjects, for there was only one Alexis St. Martin.

Although the separation of tests in man into the two kinds just described is generally valid, it implies a certain contradiction already suggested above. Tests of safety or pharmacologic action are not in themselves therapeutic or diagnostic even if they involve a drug. Patients selected as controls in therapeutic studies receive no benefit to their own health. The contradiction lies in the fact that certain authors contend that the only permissible tests are those that potentially benefit the health of every individual who participates in them. Strict application of this concept would rob therapeutic tests of much of their clinical value and scientific worth. It is true that the rewards to the participant in the two kinds of studies described above are often different, requiring different administrative approaches. However, the time has come to emphasize the basic similarity of the aim and contribution of the two kinds of study—improvement of human health. The basic similarity in risk of the two kinds of studies should be emphasized also. There is no way to exclude potential danger from any study. The simplest procedure, such as venepuncture, occasionally causes serious injury even when proper precautions are observed. However, this is no more true of studies involving pharmacology or safety than of those involving diagnosis or therapy. The probable risk must be judged for each test individually.

Codes of Practice

Most past discussions of tests in man have been concerned mainly with codes of practice. This is true of the majority of the 75 papers on clinical investigation in medicine reprinted as a single volume

* Minors, incompetents, or married women should not be accepted as volunteers or should be accepted only with caution and with the consent of their guardians or husbands, respectively.

edited by Ladimer and Newman (1963). Although stated in different ways, the papers add little to the Ten Points laid down by The Nuremberg Military Tribunal (1947) as follows:

(1) Voluntary consent of the subject is absolutely essential. Consent must be based on knowledge and understanding of the elements of the study and awareness of possible consequences. The duty of ascertaining the quality of consent rests on the individual scientist and cannot be delegated.

(2) The test should seek some benefit to society unobtainable by any other method.

(3) The investigation should be designed and based on prior animal study, the natural history of the disease or problem, and other data, so that anticipated results may justify the action taken.

(4) It should be so conducted as to avoid unnecessary physical and mental suffering.

(5) No test should be undertaken where there is reason to believe that death or lasting disability will occur, except perhaps where the investigator may serve as his own subject.

(6) The degree of risk should never exceed that which the importance of the problem warrants.

(7) There should be preparation and adequate facilities to protect the subject against even remote possibility of injury, disability, or death.

(8) Only scientifically qualified persons, exercising a high degree of skill and care, should conduct investigations on human beings.

(9) The subject should be permitted to end the test whenever he reaches a mental or physical state in which its continuation seems to him impossible.

(10) The investigator must be prepared to end the test if he has reason to believe that its continuation is likely to result in injury, lasting disability, or death.

Put more succinctly by the American Medical Association (1946), tests in man must meet three requirements: (1) There must be voluntary consent of the person on whom the investigation is to be performed. (2) The danger of each study must be previously investigated by animal experimentation. (3) The investigation must be performed under proper medical protection and management.

In the last analysis, the conduct of tests in man depends on the good sense, integrity, and freedom of the participating scientists rather than on an elaborate code of ethics. In fact, the only instances in which tests in man were grossly abused involved Nazi physicians who were under military orders to carry out experiments that by their nature excluded any possibility of voluntary participation and,

therefore, required coercion of the subjects. It is true that some irregularities of a far lesser magnitude have been alleged (Beecher, 1966) even though the scientists involved were free agents. There is no intimation that the patients or other subjects were coerced or knowingly injured in these instances, but consent either was not obtained or, if obtained, was not recorded in publication.

Any promulgation of a code or discussion of principles for tests in man is evidence for acceptance of such tests under certain conditions. It is therefore significant that in several countries codes or discussions of principles, which are basically similar and consistent, have been prepared by important persons or institutions including: Pope Pius XII (1952), the Medical Research Council (1964), the Public Health Council of the Netherlands (1957), the National Academy of Sciences---National Research Council (1965), the American Medical Association (1946), the U.S. Public Health Service (Stewart, 1966a), and the World Medical Association (1964).

Approval of tests in man is most clearly implicit in those codes of practice that set forth administrative regulations for conduct of such tests in a particular institution or in laws that require such tests. Examples are discussed in the next section.

LEGAL CONSIDERATIONS

Regulation of Clinical Research and Investigations

The U.S. Public Health Service has emphasized the importance of group review in connexion with non-standard, potentially hazardous procedures, and any involving normal subjects (Sessoms, 1958). This pattern was extended by a resolution of the National Advisory Health Council on December 3, 1965 and formalized by a policy decision issued by the Surgeon General on February 8, 1966 (Stewart, 1966a) from which the following is quoted:

> No new, renewal, or continuation research or research training grant in support of clinical research and investigation involving human beings shall be awarded by the Public Health Service unless the grantee has indicated in the application the manner in which the grantee institution will provide prior review of the judgment of the principal investigator or program director by a committee of his institutional associates. This review should assure an independent determination: (1) of the rights and welfare of the individual or individuals involved, (2) of the appropriateness of the methods used to secure informed consent, and (3) of the risks and potential medical benefits of the investigation. A description of the committee of the associates who will provide the review shall be included in the application.

It was pointed out that such a ' committee of institutional associates ' would need to be (*a*) acquainted with the investigator under review, (*b*) free to assess his judgment without placing in jeopardy their own goals, (*c*) sufficiently mature and competent to make the necessary assessment, and (*d*) composed in part of members drawn from different disciplines or interests that do not overlap those of the investigator under review. The policy was implemented by a number of administrative issuances (U.S. Public Health Service, Division of Research Grants, PPO No. 129, February 8, April 7, July 1, 1966) that established the coverage, form, and timing of the assurance each institution must furnish before it may receive additional grants or extensions of existing grants. It is required that the grantee make and keep written records of group reviews and decisions and obtain and keep documentary evidence of informed consent. The policy was also extended to intramural and contract research carried out by the Public Health Service (Stewart, 1966b).

That portion of the Public Health Act concerned with research and investigations generally (United States Code, Title 42, Section 241) requires the Surgeon General to conduct and encourage research and authorizes him, among other things to: ' Make grants-in-aid . . . ' and ' For purposes of study, admit, and treat at institutions, hospitals, and stations of the Service, persons not otherwise eligible for such treatment '.*

A Law Permitting Tests in Man

A law that permits tests in man is the 'Armed Forces Act ' of August 10, 1956 (United States Code, Title 10, Section 4503), representing the revision, codification, and enactment of pre-existing laws relating to the military services. Army Regulations No. 70-25 (Use of Volunteers as Subjects of Research, Washington, D.C., 26 March 1962) cites the 'Armed Forces Act ' as its basis. Army Regulations No. 70-25 refer only to tests on man that pertain to military research and specifically exclude ' . . . investigations involving the basic disease process or new treatment procedures conducted by the Army Medical Service for the benefit of patients.' With the exception of any specific reference to prior studies in animals, the regulations restate the Ten Points laid down by the Nuremberg Military Tribunal (see above). In addition, the regulations impose a number of other limitations, as follows: Prisoners of war will not be used under any circumstances. A physician approved

* Certain groups (e.g. commissioned officers of the Public Health Service, some merchant seamen, and others) are eligible for medical care as distinct from ' study'.

by the Surgeon General of the Army will be responsible for the care of volunteers. (The physician may or may not be project leader, but he will have authority to terminate the study at any time.) Appropriate consultants will be available at all times. No studies will be done prior to approval of each protocol by the Surgeon General of the Army and the Chief of Research and Development; for certain classes of studies, approval by the Secretary of the Army is required.

Legal and Other Requirements for Tests in Man

It is clear that all countries that are successful in developing new drugs find some way to test them in man.

United States of America

Apparently the only law clearly requiring tests in man as a condition for the licensing of new drugs is the 'Drug Amendments Act of 1962' mentioned previously. The law provides, among other things, that a license will be refused for any new drug if 'there is a lack of substantial evidence that the drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the proposed labeling thereof'. According to the law, 'the term "substantial evidence" means evidence consisting of adequate and well-controlled investigations, including clinical investigations, by experts qualified by scientific training and experience to evaluate the effectiveness of the drug involved, on the basis of which it could fairly and responsibly be concluded by such experts that the drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the labeling or proposed labeling thereof.'

The same law also provides that a licence will be refused for any new drug if the reports which are required to be submitted to the Secretary 'do not include adequate tests by all methods reasonably applicable to show whether or not such drug is safe for use under the conditions prescribed, recommended, or suggested in the proposed labeling thereof.'

Having provided that no new drug will be licensed without prior tests in man for efficacy, it conditions all such tests 'upon the manufacturer, or the sponsor of the investigation, requiring that experts using such drugs for investigational purposes certify to such manufacturer or sponsor that they will inform any human beings to whom such drugs, or any controls used in connection therewith, are being administered, or their representatives, that such drugs are being

used for investigational purposes and will obtain the consent of such human beings or their representatives, except where they deem it not feasible or, in their professional judgment, contrary to the best interests of such human beings.'

It is interesting that the law requires the consent of persons who serve as controls as well as those who take or receive candidate drugs. The fact that provision is made for professional judgment in the matter of consent is noteworthy. As pointed out by Cady (1965) the discussion of this matter of consent by the legislators who passed the law (Congressional Record, 1962) will probably be considered if the question of professional judgment in connexion with consent should ever be involved in an action in federal court.

France

The French law on new drugs (de Gaulle and his colleagues, 1959), as explained in its preamble, was made necessary by the 'Stalinon' catastrophe. This circumstance probably explains the emphasis accorded to safety in Article L. 601 of the law. The law does not specifically require tests in man, but clinical trials are required by a regulation (Article R. 5119) issued under the law. Article R. 5119 outlines a wide range of reports that must be submitted in the form of two dossiers in connexion with each application for a registration or 'visa'. Among these reports, the one on clinical trials must specify any necessary contra-indications and define the conditions of use. This regulation implies that those making clinical trials will be alert to untoward reactions during the course of the work. However, the separate paragraphs of Article R. 5119 dealing with toxicology and proof of safety do not mention tests in man. In fact, the requirement (Article R. 5.20–4) that the clinical experts will be furnished reports of all trials of toxicity before beginning clinical trials suggests that the toxicity tests are limited to animals.

An important feature of the French law is the requirement set forth in Article L. 605 that compliance of a candidate drug with the requirements of Article L. 601 must be verified by experts chosen by the manufacturer from a list established by the minister of public health and population. Article R. 5122 sets the term of office of the experts and establishes the composition of a commission to nominate them.

For further details of the workings of the French law see Guillot (1964). It is interesting that the French law and regulations apparently do not require or even mention consent by patients taking part in clinical trials.

United Kingdom

Apparently no law in the United Kingdom requires or specifically regulates tests in man. However, somewhat the same thing is accomplished by the Committee on Safety of Drugs (1965) through a ' Memorandum to manufacturers and other persons developing or proposing to market a drug in the United Kingdom '. In one sense, this brief Memorandum is broader than the American or French laws, for it refers to ' pharmacological studies on healthy human volunteers ' as a part of the preclinical investigation of a new drug. The Memorandum goes on to state that the results of clinical trials should include:

' (*a*) human pharmacological findings; (*b*) therapeutic activity; (*c*) any hazards, contra-indications, side effects and necessary precautions '.

Although the Committee on Safety of Drugs is not a part of the Ministry of Health, it is financed by the Ministry. The Committee would seem to be in an excellent position to sponsor legislation which would give protection from tort claims to the medical scientist, who carefully and properly conducts medical research on human beings in accordance with prescribed standards. Under such legislation, the acts of the scientists, within specifically prescribed limits, would be those of the government, and ' sovereign immunity ' would be waived in cases where unforeseen or unpredictable harm resulted to the subject.

Sweden

Rydin (1965) and Lönngren (1965) have discussed toxicity testing of drugs in Sweden and its relation to laws concerning drugs in Sweden. New drugs must be registered before use. The National Pharmaceutical Laboratory is in charge of the main part of the control work. The Council on Drug Acceptance is the consulting organ, and the National Board of Health determines whether or not a drug can be accepted for registration. Clinical trials include pharmacological and toxicological as well as therapeutic considerations. Emphasis is placed on individual handling of each application for registration.

Discussion

American regulations place more restrictions on the investigator of new drugs than apparently are imposed by medical ethics and custom in some other countries where greater emphasis is given to the discretion of the physician and less to the consent of the patient.

Oddly enough, a relative disregard for the consent of patients subjected to new drugs may be linked with customs forbidding all tests in man that do not contribute, at least potentially, to the health of the subject. As already pointed out, strict insistence on direct medical benefit to the patient rules out not only studies devoted primarily to pharmacology or the safety of chemicals generally but also rules out (*a*) the use of placebos in tests of efficacy and (*b*) tests directed primarily to the safety of drugs. Thus, truly scientific testing of drug efficacy would be prevented by a requirement that all tests contribute, at least potentially, to the health of every subject.

Court Action

Malpractice

Until recently there has never been a court case involving tests in man as discussed in this paper. Of course, negligence (malpractice) may be a basis for suit at any time. Since the generally accepted landmark case of Slater v. Baker in 1767, English and American courts have generally held that human experimentation is at the peril of those who conduct the tests. In the case of Slater v. Baker, tried in English courts (King's Bench) under the jury system, it was established (presumably on the assumption that the physician deviated from the then current method of treating a fractured tibia) that the physician lacked proper skill and failed to disclose his intention or to obtain the patient's consent in using a previously untested procedure. The case established a precedent which has been followed in English and American law and, in various degrees, in other parts of the world.

In any case in which negligence is alleged, the true issue of investigation does not arise. Should a claim arise in the course of a planned test in man, the best defence is proof that the subject assumed the risk knowingly and voluntarily (Monthly Summary from the Office of General Counsel, U.S. Department of Health, Education, and Welfare, December, 1959). Certainly, the mere fact of investigation is no longer considered evidence of negligence. As Cady (1965) has pointed out, the question is not whether a study is made but how well it is made.

In France, a physician found guilty of malpractice may be subject to fines or a prison sentence under Articles 319, 320, and R.4, 4th paragraph of the penal code or to damages under Article 1382 of the civil code.

A Case Involving Scientific Tests in Man

The only case brought to trial involving scientific tests in man was the matter of Hyman v. Jewish Hospital (15 NY 2d 317) in which Hyman, a member of the board of directors of the Jewish Chronic Disease Hospital, sought the right to examine the records of 22 patients who had taken part in a test. The petition was granted by the trial court, reversed by the lower State appeals court, but finally upheld by the Court of Appeals of New York, the highest court in the State. The final decision ruled that the director was entitled to examine the medical records in connexion with the study because of the possibility of liability of the Hospital, though no opinion whatever was expressed on whether such liability would indeed exist.

The court contest attracted interest in the case from the New York State Department of Education, and the professional conduct of the two physicians primarily concerned with the study was reviewed by the Regents of the University of the State of New York acting under their responsibility for licensing the medical profession. The findings of fact in the case were published by Freund (1965) and discussed at length by Langer (1966). Langer (1966) has also reproduced the body of the Regents' decision. Briefly, the study involved subcutaneous injection of living cancer cells into 19 very debilitated patients suffering from non-malignant chronic disease and into three equally debilitated controls who had cancer. The patients were told that a cell suspension would be injected as a test for immunity or response. The patients were also told that within a few days a lump would form at the site of injection, but that it would gradually disappear after a few weeks. This is exactly what happened. The record indicates that all the patients approached agreed to the injection and, further, that none suffered any ill effects other than the transient discomfort of the injection and the nodule it produced. The study showed that the sick and debilitated patients with non-malignant disease rejected cancer transplants as promptly (6–8 weeks) as healthy volunteers who had been tested earlier. The test also confirmed that cancer patients are deficient in their ability to reject cancer transplants, the rejection requiring six weeks to several months. The court noted that these findings open up the possibility of stimulating defence against cancer either before it strikes or perhaps even later when it has taken hold.

In spite of these considerations, the Regents found the two physicians guilty of ' unprofessional conduct ' and of ' fraud and deceit in the practice of medicine ' because the patients had not been told that living cancer cells were to be injected and, therefore, the patients could not give informed consent. The physicians' medical

licences were suspended for a year, but the sentence was stayed, and the men continued to practice while on probation for one year. The Regents held that (*a*) it is the volunteer, and not the physician, who has the right to decide what factors are or are not relevant to consent—hence all details must be revealed—and (*b*) when acting as an investigator, the physician has no claim on the doctor–patient relationship that in a therapeutic situation would give him the generally acknowledged right to withhold information if he judged it in the best interest of the patient. The desirability of obtaining consent in writing was emphasized.

Perhaps the most important aspect of the entire proceedings was that the legality and propriety of the study itself were never challenged; only the failure to obtain informed consent was condemned. This is a strong indication of the public support which medical research enjoys. Further evidence may be found in the informed and constructive comments on the case which appeared in some parts of the lay press (Carley, 1966) as well as in some scientific journals (Freund, 1965; Langer, 1966; Halpern, 1966).

DESIGN OF STUDIES

Parameters of Study

Tests in man should be carried out only after whatever tests may be possible in animals have revealed one or more parameters suitable for study in man. Even the most superficial study in animals will often reveal which organ system is involved predominantly in toxic action. One may then determine by further study which of the functional tests appropriate for exploring this organ system in man is best able to measure the alteration produced in animals. Thus, an index test may be found. Emphasis should be placed on the appropriateness and depth of the studies, not on the use of large numbers of animals. Every effort must be made to avoid producing in man any clinical illness or any irreversible subclinical change. Therefore, emphasis must be placed, in preliminary animal studies, on finding tests to detect the earliest, mildest, reversible change that indicates action of chemical under study. A valuable discussion of tests of the various organ systems of man is given in a book entitled *Animal and Clinical Pharmacologic Technique in Drug Evaluation* edited by Nodine and Siegler (1964). Even a complete listing of appropriate tests is beyond the scope of this paper, but the following illustration may be helpful.

Research has failed to reveal a test for any effect of organic phosphorous or carbomate insecticides that is more sensitive than a test

for depression of blood cholinesterase activity. This is true even if the comparison is made with alterations of conditioned reflexes (Letavet, 1961) or other behavioural change (Carpenter and his colleagues, 1961). All potential insecticides that produce neurotoxicity (delayed, irreversible paralysis) also produce cholinesterase inhibition. Thus, measurement of cholinesterase activity should be used as an index test in any investigation in man concerned with an organic phosphorous or carbamate compound. However, at least one organic phosphorous compound (tri-p-ethylphenyl phosphate) that is not insecticidal and does not inhibit cholinesterase, is neurotoxic in chickens (Cavanagh and his colleagues, 1961). Clearly, the possibility that an organic phosphorous compound is neurotoxic should be thoroughly studied in chickens before it is tested in man.

Some functions may be measured mechanically rather than biochemically. Complex reaction time was found by Durham, Wolfe and Quinby (1965) to be affected in persons sufficiently exposed to organic phosphorous insecticides. It seems reasonable that the same would be true of airway resistance. Depth perception was affected by traces of tetraethylpyrophosphate in one eye, but not in both eyes (Upholt and his colleagues, 1956). In general, even those functions that are relatively simple to measure mechanically in man are difficult or impossible to measure in animals and yet, like the ones just mentioned, they may be critical to the safety of workers or others.

An effort should be made to measure the effect, if any, of a compound on the critical functions of those who may use it even though there is no pharmacological reason to think it will affect those functions. For example, drugs or other chemicals to which pilots may be exposed should be tested for their effect on vision and reaction time.

Any test of pharmacological action is of potential value in judging the safety of a chemical not intended for use as a drug. No action is expected under ordinary conditions of use, and if one occurs, its significance must be evaluated. On the contrary, pharmacological action must be present if a compound is a drug. Thus, a drug is judged unsafe only if it leads to discomfort or dysfunction incommensurate with the benefits it produces.

Whenever either drugs or other chemicals are tested in man, an effort should be made to measure the compounds or their metabolites in blood and excreta. Such measurements permit use of information gained during the test in the solution of diagnostic and other practical problems. For example, if a person is suspected of being poisoned by a particular compound, it is of great interest to

know whether the concentration of it in his blood is merely equal to or is significantly greater than that previously found in healthy volunteers or workers. By the same token, it is valuable to learn as much as possible about the metabolism of compounds in man. Information on this matter may help to explain observed similarities or differences in the pharmacological or toxicological action of the compound in man and animals (Brodie, 1964) and such information may help in selection of the best species of experimental animal for further exploration of the compound. It often happens, not only in connexion with metabolism but also in connexion with other parameters, that studies in man will raise questions that can be explored best in animals before further evaluation in man.

No matter what special parameters are selected for study, every test in man must include a medical history and general physical and laboratory examinations. These studies must be made before the chemical under investigation is administered, and they must be repeated at suitable intervals. This general surveillance should detect unexpected reactions including sensitization that may be peculiar to man. The surveillance should also detect illness that is aetiologically unrelated to the chemical under study. Such basically unrelated illness may influence the reaction of the body to the compound under study or, if not detected early, it may serve to confuse the interpretation of the results.

Selection of Dosage

The highest dosage selected for test in volunteers should be one which is believed to be (*a*) harmless on the basis of animal studies and all other available information, and (*b*) capable of influencing some measurable parameter. For compounds of low toxicity, measurement of excretion of the compound or its metabolites may be the only parameter influenced. For compounds of moderate or high toxicity, it may be desirable to use a graduated series of dosages beginning with a very low one and gradually approaching the highest dosage that produced no significant injury in animals.

Regardless of the toxicity, it is usually desirable to have more than one dosage either in a single test or in succeeding tests in order to determine whether responses are dose-related. Even if dose-response is not under investigation, it is necessary that a control group be included in every study. Its use may serve to detect laboratory error and other factors that might influence the results without having any relation to the agent under study.

In general, the dosage is not subject to control by the investigator in tests carried out on workers who are occupationally exposed to a

chemical. Therefore, measurement of the compound or its metabolite(s) is of paramount importance in such tests. If the dosage is unknown, it may not be possible to determine what proportion of the compound or metabolite(s) is actually detected. Even so, it may be possible to show that the results are dose-related by comparing them with results of environmental measurements. No matter what proportion of compound or metabolite(s) is detected, the results may be of great value in determining whether a person exposed under quite different conditions but tested under the same conditions has absorbed more or less of the compound than the workers.

Choice of Subjects

Subjects for study may be patients, volunteers, or workers.

Patients

Usually there can be only limited choice of subjects in connexion with tests of the effectiveness of drugs. All subjects, including the controls, must have the condition that the drug under study is supposed to ameliorate or cure. The only exception involves those curable diseases which can be produced in man at will. Even in this instance, the disease usually will not be produced unless spontaneous cases are not otherwise available and study of the disease is imperative. Malaria may be the only example. Appropriate initial studies of malaria therapy are difficult or impossible in the tropics where the disease is common. Some therapeutic studies are meaningful only if carried out on persons whose mode and duration of infection are uniform. Because of these considerations and the tremendous importance of the disease, many studies of antimalarial drugs have been carried out on volunteers who were purposely infected with a selected strain of malaria (Coatney and his colleagues, 1953; Goodwin, 1952).

Volunteers

Selection of volunteers is determined by (*a*) the number of people required and (*b*) the duration of the study. People from any group including the general population may be recruited for short periods of testing, especially if the tests involve little inconvenience. In studies in which each individual need be tested only once, and that only briefly, one could eventually study large numbers of people enrolled from the general population. However, for studies requiring a substantial number of volunteers for an extended period, the only practical method is to recruit volunteers through employment or

through appeal to some special interest of the candidates. Thus, depending on the nature of their interests, volunteers for studies of malaria and most studies of safety may involve the following groups: (*a*) patients; (*b*) fully paid employees from the general population, groups of workers, or other groups; (*c*) laboratory staff; (*d*) medical or other students; (*e*) military personnel and conscientious objectors; (*f*) persons with some occupational interest in the problem under study; and (*g*) prisoners.

If patients wish to volunteer for a test having no relation to relief of their own condition, they may do so provided they are capable of giving consent. In fact, there are a few studies that can be carried out in man only in patients. An example would be measurement after death of the distribution of a chemical in all tissues of the body. Another example would be any study involving debility or a specific metabolic disorder as a necessary condition.

In any study of patients as volunteers, the investigator should not be the personal physician for any volunteer, and a personal physician as well as the investigator should be able to discontinue the participation of one or more volunteers, as required. Of course, each patient should be able to stop his own participation at will.

Fully paid employees have been utilized in small numbers and usually for special purposes. Although perhaps not typical, Alexis St. Martin, who was studied by Beaumont (1833) is the most famous. This method of obtaining volunteers deserves more consideration. It offers a particularly good opportunity to choose the desired distribution of persons of different age and sex.

A number of studies have been carried out using laboratory staff. This method has the advantage that the volunteers are conveniently available for repeated brief examinations, which under other circumstances would require far more of their time and of the investigator's time. Another advantage is that such volunteers, because of their technical background, are in an unusually good position to understand the nature of the test and its potential risks. This means that the investigator need not exaggerate the danger of the study in order to be sure he has not underestimated it. The chief disadvantage of using staff as volunteers is that it is difficult to avoid subtle coercion. Many staffs are not large enough to provide a numerically adequate group for study even if everyone participates. The result is social pressure to be one of the group. The pressure may increase as the study progresses and the volunteers develop an *esprit de corps*. By the same token, a volunteer cannot leave such a study without attracting special attention. There is a certain danger that because of their social and economic relations to the investigator, these

volunteers may be more likely than some others to behave as they think they should. Obviously, this danger is greater in connexion with subjective tests and should be essentially non-existent in connexion with chemical or physical measurements. All the disadvantages of studies using laboratory staff are accentuated in studies in which the investigator is also the subject.

The comments in the preceding paragraph apply with equal force to medical or other students used as volunteers. This is especially true if the students come from the same school or department as the investigator. The disadvantages would be lessened if the volunteers were drawn from a large student body so that, although the participants are known to one another, they are not generally recognized as a group by the student body.

The use of military personnel or civilian personnel of military installations in medical studies is limited to the armed forces and to the problems peculiar to their mission.

People may volunteer for tests because of an occupational interest in the problem under study. In this respect, they are like military volunteers, because they know they may contribute directly to their own future welfare if they can help in the solution of a problem peculiar to their work. For example, crop duster pilots volunteered during the winter season when they were not flying for a study to learn how tetraethylpyrophosphate affects vision, and especially why, when pilots had got it in their eyes, it had interfered with their ability to judge distance and, thus, to control their planes (Upholt and his colleagues, 1956).

Prisoners offer many advantages as candidates for medical study. The population of a single prison is often large enough to permit an adequate number of volunteers of the desired range of age. In many instances, both men and women will be available through the same prison administration, though seldom at the same installation. If the institution is large enough, sufficient volunteers may be obtained without exerting any social pressure on them, and a volunteer can stop participating without drawing special attention from his fellow prisoners. With proper scheduling, the studies may be carried out with efficient use of the investigator's time and without interfering with the prisoners' regular duties. The disadvantages of using prisoners include their erratic motivation and their often considerable variation in ability to understand the nature and risks of a study. A major disadvantage is that all doses or other treatments must be given under observation. By contrast, staff members not only may take their own doses but may do so during travel or other inconvenient circumstances.

Those without personal familiarity with tests in man sometimes express doubts that volunteers act without some form of coercion. The doubt is probably most often expressed about prisoners but may be referred to medical students or any other group from which volunteers may be drawn. The most convincing argument against this sort of doubt is active experience with a properly conducted study.

Workers

In the preceding section on volunteers, workers might be included under several of the headings, especially 'fully paid employees'. The distinction between participation of an employee in a study as a volunteer or as a worker lies in the nature of the exposure. Regardless of his employment status, a person is a volunteer if he agrees to receive an exposure administered for the purpose of making a study. On the contrary, a worker is not a volunteer in the usual sense if he agrees to undergo medical study in relation to an exposure or some other factor that is a necessary condition of his employment.

One might ask how participation of a worker in the kind of tests under discussion differs from that ordinarily involved in good industrial medicine. The difference is one of emphasis; there is no clear dividing line. Tests in man emphasize our ignorance of how chemicals or other environmental factors act in man; hence, these tests seek new information. They seek safety by trying to identify and define unexpected dangers. Industrial medicine emphasizes our wealth of knowledge of how chemicals or other environmental factors act in man. Like any good medical practice, industrial medicine attempts to prevent—and if necessary cure—injury by taking all known dangers and signs of danger into account.

Some opposition to tests in man seems to be based on a desire of industry to limit the extent of tests of whatever nature that are required for official acceptance of a product. This opposition might be reduced if it were understood fully that many of the tests that need to be done regarding pharmacology and safety can be carried out in workers.

It is a curious fact that, in studies of workers, no question is ever raised about consent to exposure although consent for diagnostic procedures should be obtained by the investigator. It is assumed that consent for exposure exists if a person is employed in the manufacture or formulation of a chemical provided the management takes conventional measures to limit exposure and no illness occurs as a result of the exposure. This is true even in those instances in which the amount of chemical absorbed is equal to or greater than that adequate for studies in volunteers.

The major disadvantage in using workers for tests is the difficulty in regulating or even measuring the dosage. However, this is not a handicap in many biochemical studies, especially where the results can be related to measurement of the compound or its metabolite(s) in blood or excreta. Furthermore, if the compound or its metabolite(s) can be measured, the results of the study can be used directly in interpreting the safety of other persons exposed under different circumstances that lead to the same--or different--blood levels or excretory levels.

Often the ideal scheme is to use volunteers to relate carefully measured dosages to the dynamics of storage and excretion and then to carry out other studies, especially prolonged ones, in workers. Certainly, the exposures of workers often last much longer than those of volunteers.

Protocol and Conduct of the Study

Function and Content of the Protocol

The plan for every study in man should be put in writing. Before this is done, it may be well to reach an informal agreement with the administration of any institution where the study is to be made. Such preliminary discussion may save a great deal of inconvenience by bringing to light requirements of the institution that, although of no scientific importance, may be of crucial practical significance. In general, the writing of a protocol forces the investigator to face not only the scientific but also the technical and administrative logistics of the study. Once the document is prepared, it may be used as a basis for approval of the project by (*a*) custodial authorities for administrative feasibility, or (*b*) academic or comparable authorities for scientific merit and safety.

The protocol should cover at least the following topics: (*a*) summary of the problem which justifies the study; (*b*) specific purpose of the study; (*c*) experimental design; (*d*) safety of the study; (*e*) rewards to volunteers; (*f*) proposed contract or other form to document consent.

The ' problem ' referred to in item (*a*) may be very broad. It is often ill-defined, but never unimportant. An example would be hypertension. The fact that hypertension may have multiple causes, none of which are fully understood, does not detract from its importance as a medical problem.

By contrast with the problem, the purpose of any study in man should be clearly defined and is frequently very limited. Thus, study of a candidate antihypertensive drug might be limited to investiga-

tion of its metabolism in normal people and those with high blood pressure.

The section of the protocol dealing with experimental design should state the number of people to be studied, their distribution into experimental groups, the rate and duration of dosage, and the observations to be made. The groups should be large enough to permit statistically valid conclusions even if some of the participants drop out before the study is complete. The protocol should state clearly what measurements are to be completed before dosage or some other experimental situation is instituted, and how long this preliminary period will be. The protocol should also state what observations are to be made after dosage is discontinued. The duration of study need not be predetermined, but the schedule may be defined in terms of some pharmacological constant. For example, the study may be continued after dosage is stopped until the excretion of a metabolite of the chemical under study reaches the level found in the controls.

The section on experimental design should give particular attention to things that concern the volunteers directly. Thus, the protocol should state the kind and approximate number of samples which will be taken from each person. It is just as important to enumerate procedures that may involve inconvenience or interference with institutional routine as it is to define those that may involve pain, discomfort, or even risk. In our experience, we almost lost a project because the way in which urine samples were collected interfered with the employment of a few of the volunteers in a weaving mill, which was a part of the occupational training and therapy programme of the prison. Fortunately, it was possible to change the schedule for collecting samples without decreasing their number or scientific value, and the study was saved. Not all difficulties can be foreseen, but they can be reduced by careful planning.

The section on safety is one of the most important parts of the protocol and should be brief and inescapably clear. The major points may be supported by published reports of animal experiments or by other appropriate documents presented as appendices. In any event, this section must define: (*a*) the nature of any hazard; (*b*) the probability of danger; (*c*) the means available to detect, or if possible, predict injury; and (*d*) the possibility of specific treatment if injury should occur. The section on safety must include a statement that individual volunteers of the entire group will be withdrawn from active participation if circumstances require it. Of course, a volunteer whose active participation is stopped will continue under observation, and he will receive therapy if needed.

The protocol should specify the rewards (e.g. money and a certificate) the investigator is prepared to give the volunteers. It may list other compensations that would be appropriate although they are not under the control of the investigator. For example, if the work is done at an institution which is able to offer some other form of reward, then that, too, should be mentioned. Some prison systems routinely give a reduction in sentence for good behaviour, including but not restricted to participation in medical research.

The last section of the protocol should refer to the agreement or contract to be used in connexion with the study. The American Medical Association (1961) has supplied a model release form (Form 29) for use with investigational drugs (*Figure 1*).

AUTHORIZATION FOR TREATMENT WITH DRUG
UNDER CLINICAL INVESTIGATION

Date ..... *Time* ..... *a.m./p.m.*

I authorize Dr. ....., the attending physician, to treat ..... with the drug presently identified
(*name of patient*)
as ..... for the following condition .....
.....
(*Describe symptoms of disease to be treated*)

It has been explained to me that the safety and usefulness of the drug in the treatment of patients for the above condition are now being investigated and that the manufacturer or distributor has supplied the drug for the purpose of providing further evidence of its safety and usefulness.

I voluntarily consent to treatment with the drug and release the attending physician from liability for any results that may occur.

Signed .....
(*Patient or person authorized to consent for patient*)

Witness .....

Figure 1. Consent form

The author and his colleagues have used a more elaborate contract in connexion with studies in institutions. The contract includes the following features:

(1) Date.
(2) Name of organization (or persons) making the study.

(3) Name of institution where study is made.

(4) Name of applicant.

(5) Description of the study.

(6) Kind and approximate number of services applicant will be expected to contribute and the approximate duration of service.

(7) Statement by the applicant that the procedure, value, and danger of the study have been explained to him and that he is fully aware that there can be no guarantee that he will not become ill (in a study in which illness is really anticipated, its nature should be defined in the contract).

(8) Statement by the applicant that he knowingly and voluntarily accepts the risks, and agrees to co-operate in the work.

(9) Amount of money and other rewards the applicant will receive in exchange for his services provided his participation is satisfactory.

(10) Applicant's statement on behalf of himself, his representatives, and heirs that he releases the investigators and the organization they represent from all liability, including claims, and suits at law or in equity for any injury, fatal or otherwise, that may result from his participation in the investigation.

(11) Signature of the applicant.

(12) Signature of witnesses.

(13) Signature of responsible investigator who accepts the applicant as a volunteer.

(14) Signature of officer who approves contract for the institution where the work is done.

NOTE.—The contract always omits any statement that the applicant agrees to remain in the project. In some instances, the signatures mentioned above were followed by a statement signed by an officer of the institution: ' I attest that the purposes, procedures, and inherent risks of this study were fully explained to the applicant by the officer in charge of the investigation. I am convinced of the applicant's complete understanding of the study and of his willingness to participate in it. I am confident that no duress of any kind was present in the proceedings leading to the signing of this contract '.

Initiation of a Study

The method of obtaining volunteers must vary with circumstances. If the study involves members of a laboratory staff, they will be generally familiar with the need for the study and the character of the risk, if any, as the preliminary work progresses in experimental animals. If the work is to be done with a group of people who have no preliminary introduction to it, it is best that a completely imper-

sonal notice be issued and that the candidates consist only of those persons who show enough interest to seek further information. Such a notice should state briefly the purpose and nature of the study and invite anyone interested to attend a meeting at a specified time and place at which a further explanation will be offered. If the work is done in a prison, the custodial staff should screen those who propose to attend the introductory meeting in order to exclude those who are unsuitable by reason of a short sentence, a reputation for making trouble, or some special duty which would conflict with participation in the study.

At the meeting, the principal investigator and others if necessary should explain the study in detail. Emphasis should be placed on: (*a*) the value of the work; (*b*) what each volunteer will be asked to do; and (*c*) the hazards, no matter how remote, that the volunteer may encounter. Of course, the rewards, the agreement, and other administrative arrangements should be explained. When questions from the candidates have been answered, the candidates are asked whether they wish to volunteer. A list is made of those who do, and they are instructed when and where to report for preliminary medical examination.

The preliminary examination consists of a complete medical history and appropriate physical and laboratory examinations of each applicant. The examination also involves special tests, including the index test, dictated by the nature of the study. One or more parts of the preliminary examination may serve to exclude some applicants from the study and all parts are used as a base line for judging future findings in the others. Those candidates who are found completely satisfactory are asked to sign the agreement or contract, and they are not considered volunteers until they have done so.

Although candidates for a study should be told its complete design, it should be understood that no volunteer is to be told the dosage group to which he belongs. A useful device is to give placebos to all dosage groups for about a week at the beginning of a study. Appropriate samples are taken during this period when each volunteer serves as his own control. It sometimes happens that more complaints are received during the period when placebos are given to everyone than during the remainder of the study. This is entirely understandable, because the volunteers have been told about risks which the investigator felt morally obliged to mention although he considered that the chance they would materialize was really nonexistent. Furthermore, it is fair to say that volunteers think that their appetite or general health has improved about as often as they think they have suffered some injury. In any event, it is absolutely

necessary to have one or more control groups throughout the study. Such a group permits a random distribution of complaints which have no real connexion with the experimental conditions. It also permits immediate recognition of the cause if some unexpected finding is the result of laboratory error. Some measurements tend to vary even when made in the best laboratories. An example is the potentiometric measurement of blood cholinesterase. If it is found that the blood cholinesterase of all groups including the control, drop sharply for a day or two and then return to normal, the result may be safely ignored as being unrelated to the variable under study.

Double Blind Studies

The double blind technique requires that neither the subject nor the investigator responsible for recording their reactions be aware of which volunteers receive different chemicals or different dosage levels (including placebo) of the same chemical. It follows that a third party will be responsible for assigning dosages but have no direct contact with the volunteers during the course of the study. The ' third party ' is usually a member of the team of investigators; he may take major responsibility for design of the study and interpretation of the results but not for collection of the data.

The study may involve compounds (usually drugs) of which one has well-known effects in man and the other has been shown to be similar both qualitatively and quantitatively on the basis of chemistry and animal tests. Under these circumstances, there is no objection to the double blind technique and, in fact, its use is important, particularly if the responses are mainly subjective. However, the value of the technique in many situations should not lead to a routine demand for it in all studies. Need for the technique diminishes as the degree of difference to be measured increases. Furthermore, the need is inversely proportional to the objectivity and repeatability of the measurements required. Finally, the double blind technique is contra-indicated in connexion with early studies of really new materials. Under these circumstances, the investigator should have every opportunity to discontinue or otherwise modify the study in response to the first hint of injury.

Contrary to the opinion of some authors, use of the single or the double blind technique has no necessary bearing on the matter of consent. Candidates for studies by the auther and his colleagues were always told what dosage levels were planned, including the fact that some volunteers would receive a preparation containing none of the chemical under study. The only information withheld was

who got which dosage. The candidates were told not to volunteer unless they were willing to receive the highest dosage.

Closing Ceremony

It is almost always wise to have a closing ceremony to recognize the contribution of the volunteers. The main thing is to say ' thank you ' and to emphasize the contribution of the volunteers toward solution of a scientific problem. It has been our custom to present an engraved certificate (*Figure 2*).

Department of Health, Education, and Welfare

Public Health Service

presented to

(FULL NAME)

in grateful appreciation of services as a volunteer in a research study of broad significance to the advancement of medical science and the ultimate benefit of mankind

Seal

....

....

Signatures *Signatures*

Figure 2. Certificate

The certificate is signed by the Surgeon General and other important persons.

Protection of the Volunteer

Protection of the volunteer—to summarize what has already been said in other contexts—includes:

(1) Guarantee that no study is made without valid consent—that is, continuing consent freely given by a person legally capable of giving it. The recognized ability of the volunteer to withdraw from the study at any time even without reason is a part of this concept.

(2) Qualification of the investigator.

(3) Freedom of the investigator to stop the participation of volunteers if he thinks their continuation in the project might injure them.

(4) Care in the event of injury. Tests in man should not be done in situations that do not permit medical care of the volunteer if he becomes ill. For some large medical institutions, this presents no problem. For example, provision for care of whatever duration necessary is made in the Army Regulations cited above. For smaller

institutions, insurance may be the best method to prepare for the unlikely eventuality of injury. Zavon (personal communication) reported that, in a study of the irritant effect of air pollution on the eyes, insurance for $3 million was purchased for $278·90.

Protection of the Investigator

Protection of the investigator should be built into the design of every test in man. Of course, the most important protection of the investigator consists of demonstrable evidence that he has done everything possible to protect the volunteer.

Proof of Consent

As we have seen, the matter of consent was the only thing brought into question in the case of Hyman v. Jewish Hospital (15 NY 2d 317). Proof of consent involves not only evidence of the fact but also evidence that the volunteer was legally capable of giving consent. The fact of consent is best established by a written document as discussed on pp. 219–220. While written consent will not protect the investigator against liability for his negligence, it will constitute a defence against claims for injuries resulting from foreseeable and assumed risks (Monthly Summary from the Office of General Counsel, U.S. Department of Health, Education, and Welfare, December 1959).

Proof of Qualifications

Any investigator or team of investigators who undertakes tests in man should be fully qualified not only from a general medical standpoint, but also from the special standpoint of the problem under study. Evidence of qualification may include consultantships, membership in learned societies, and papers published in the field. Proof of qualification for a particular study is strengthened by a signed permit from the investigator's institution and/or another agency involved. Such approval would, of course, be conditioned on other relevant factors as well as the qualifications of the investigator.

Proof of Value of the Research

In many instances the value of research on man is evident to a layman and requires no justification. Even in such instances, the reason should be outlined clearly in the protocol. Again, official approval offers additional evidence that the work is considered valuable. Because the word 'experimentation' carries the legal

implication of malpractice, it is only common sense to avoid its use in connexion with proper medical investigation.

Insurance

Insurance is not only a protection for the volunteer but also for the investigator. Ballard (1964) has discussed the possibility of legal and financial support from the manufacturer of a compound in the event that an authorized investigator of the compound is sued.

Tort Claims Act

Apparently no tort claims act has been passed for the protection of the medical profession specifically. Certain other groups enjoy this benefit. Basically, such an act would provide that a government will defend suits for claims arising from actions of their employees within the scope of their employment as defined in the act, and that if judgment is rendered against the scientist the government will pay the judgment. Although the medical and paramedical personnel directly affected by such an act would be those employed by the government, the act would influence relevant legal action at all levels. The careful delineation of duties—including the necessity to obtain informed consent—associated with research as well as with diagnosis and therapy would not only protect the investigator acting within the limits of his employment, but it would be a substantial evidence of confidence in medical research in general.

MOTIVATION OF VOLUNTEERS

Different people participate in research for different reasons or different combinations of reasons including: (*a*) tangible rewards, (*b*) a desire for new experience, (*c*) occupational or other specialized interest in a specific project, (*d*) interest in science generally, and (*e*) a desire to contribute to society.

Tangible rewards may include money and (in the case of prisoners) reduction of prison term. Reduction of sentence may be guaranteed or potential, that is, it may be prorated according to the duration of each volunteer's participation in the study, or it may consist of the hope that participation will influence parole or the dropping of detainers by another court. Although tangible rewards may be critical for participation in some instances, the importance of this factor is easily overestimated. We have carried out a number of studies successfully even though we could afford only token payments. We have recruited prisoner volunteers without difficulty when no reduction in sentence could be offered.

A general interest in science is probably the most important general reason leading people to become volunteers in medical studies. Many people want to help in solving any problem they understand. If the problem involves their occupation, there is every reason they will understand its importance and they may even have some technical insight into it.

Of course, prisoners share with people in general an interest in science, a need for money, and a desire for new experience. In fact, the desire for new and varied experience may be especially strong in people who are confined. However, the main feature that distinguishes prisoners from others in this connexion is an unrecognized urge to do something useful. Either on a conscious or subconscious level, many prisoners wish to compensate for records indicating little positive contribution to society and, consequently, little positive recognition from society. Few prisoners will admit this urge to contribute except in time of war when any worthwhile act can be attributed to patriotism. A partial exception was revealed by one prisoner who told me that participation in the project had led to his reinstatement in his family. His father was pleased that he had finally done something commendable. Even though prisoners generally deny it, the author believes that an urge to serve society is the most important single motivation for their participating in projects.

Continuation in a project requires an element of steadfastness which is not necessary for initial recruitment. This steadfastness seems to be characteristic of the individual. It has little or no relation to the nature of the study or to whether discomfort is or is not involved. Dropping out, which is freely permitted in our studies, usually occurs very early in the project, frequently before anything has really happened at all. Once a project has settled down to a routine, it is most unusual for a volunteer to drop out. In studies such as those on antimalarial drugs in which production of the disease was a necessary part of the work, the men accepted their illness with true heroism.

CONCLUSION

The limited ability of animal tests to predict human reactions to chemicals is recognized generally. Tests in man represent a logical step to full-scale use of a chemical. The fact that even hypersensitivity is basically dose-related emphasizes the importance of a continuing surveillance of workers or other persons whose exposure is intensive and prolonged in order to detect idiosyncrasy as well as overt toxicity at the earliest possible moment.

Many countries accept the necessity for orderly testing of the effectiveness of new drugs in man. Such testing of drugs for effectiveness implies, but does not require, that the safety of therapeutic doses will be observed critically, and that some pharmacological studies of the drug will be made in man. Furthermore, testing for effectiveness does not necessarily mean that possible adverse reactions will be looked for systematically after a drug is released for prescription use. Thus, studies of the safety of drugs in man often are not required by law or custom in exactly the same way that tests of effectiveness are required. There is even less recognition of the need to test in man the safety of other chemicals to which people will be exposed. In fact, little thought seems to have been given to the question of whether it is ethical to release a new chemical for general use before it has been tested under controlled conditions in a limited population.

If it is decided that general release of a new chemical under conditions that defy orderly study is unwise—or, in fact, if studies of therapeutic effect are to be done scientifically using proper controls—then we must abandon the notion that tests in man can be justified only if they are of possible direct benefit to the person tested. Countries apparently vary widely in the tenacity with which they support this notion. Certainly, tests of great value to society have been carried out without injury, but also without any medical benefit to the volunteers involved. However, only in special situations, especially the military, are such studies clearly permitted by law.

In the last analysis, the conduct of tests in man depends on the good sense, integrity, and freedom of the participating scientists rather than on an elaborate code of ethics. Even so, most important progress could be made by passage of a tort claims act which would carefully define standards of conduct for investigators in such a way that any scientist who complied with the standards would be within the scope of his employment and, therefore, under the protection of the law. The medical profession should sponsor passage of such laws.

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PCB Metabolism in Rats Following Prolonged Exposure to Aroclor 1242 and Aroclor 1016

by VIRLYN W. BURSE
Center for Disease Control
3100 Clifton Rd.
Atlanta, Georgia 30333

ROBERT F. MOSEMAN and G. WAYNE SOVOCOOL
Pesticides and Toxic Substances Effects Laboratory
National Environmental Research Center
Environmental Protection Agency
Research Triangle Park, North Carolina 27711

and

ELLEN C. VILLANUEVA
The Coca Cola Export Corporation
260 Peachtree, N.W.
Atlanta, Georgia 30303

Determination of the level of metabolic products present in urine has been utilized as an indicator of type and degree of exposure to chemicals. Two of the most widely studied urinary metabolites, namely DDA, the carboxylic acid metabolite of DDT and the para-nitrophenol metabolite of parathion have been used to gain insight into the degree of exposure to these parent compounds (LAWS, et al. 1967; ELLIOTT, et al. 1960).

Polychlorinated biphenyls (PCB's) were first reported as environmental contaminants in 1966 (JENSEN). The scientific literature now abounds with reports concerning the analysis and toxicology of these highly stable and persistent materials.

For many years it was generally believed that PCB's were not metabolized to any appreciable extent by mammalian systems (REYNOLDS, 1969). However, as early as 1959, BLOCK and CORNISH (1959) reported on the conversion of biphenyl and 4-chlorobiphenyl to monohydroxylated compounds in the rabbit. WEST, et al. (1956) isolated pure compounds resulting from the metabolism of biphenyl in the rat. Recently JENSEN, et al. (1974) described the separation and identification of individual isomers of PCB's in the technical material and in human adipose tissue. Based on the percentages of various isomers stored in adipose tissue, he indicated that two adjacent unsubstituted carbon atoms were required for rapid metabolism. This supported the contention of previous investigators who were working with chlorobenzenes (JONDORF, et al. 1955). KAISER and WONG (1974) reported that microbial degradation of Aroclor 1242 resulted in non-chlorinated aromatic and aliphatic products. GRANT and his group (1974) demonstrated that Aroclor 1254 was metabolized in rats. He noted significant differences in GLC peak patterns for the PCB standard and the PCB which was extracted from the tissues. Similar finds were reported for Aroclor 1254 by CURLEY, et al. (1971) using Electron Capture-Gas Chromatography in their examinations of rat tissues and urine. No metabolites were isolated or identified.

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This paper reports on metabolic products observed in rat urine, through Coulson Conductivity-Gas Chromatography (CC-GC) and combined Gas Chromatography-Mass Spectrometry (GC-MS) analyses, following a prolonged diet of Aroclor 1016 or Aroclor 1242.

EXPERIMENTAL

Aroclor 1016 and 1242 were of electrical grade, lot No. KB-06-756 and KB-05-415, respectively. They were supplied by Monsanto Chemical Company, St Louis, Missouri. The Aroclors were fed in parallel experiments to male Sherman strain rats, 61 to 73 days old at a dietary level of 100 ppm each. Twenty-four hour urine was collected from four experimental rats fed Aroclor 1242 or Aroclor 1016 and one control under the following schedule: 2 weeks after onset of the experiment; one and 2 months after onset of experiment; 4, 6, 8 and 10 months after onset of experiment. After 6 months on the diet, some rats were allowed to recover by removal of PCB diet, for time periods of 2, 4 and 6 months. Urine was collected at these intervals. A more detailed accounting of the experimental protocol can be found in another publication (BURSE, et al. 1974). The amount of Aroclor 1242 consumed ranged from 6.6 mg/kg bodyweight/day to 3.89 mg/kg bodyweight/day while Aroclor 1016 ranged from 6.9 - 3.5 mg/kg bodyweight/day.

The twenty-four hour urine samples were combined from four rats in each experimental group. Total urine volume ranged from 40 to 80 ml. The urine was refluxed in an equal volume of concentrated HCl for three hours and extracted 3 times with 50 ml of benzene. The benzene extract was washed with 20 ml of 5% NaOH w/v followed by 20 ml water. The aqueous phases were combined and acidified with 20 ml of 1.2 N HCl. The acidic aqueous phase was extracted 3 times with 10 ml of benzene and dried over sodium sulfate. To each sample was added diazomethane (STANLEY, 1966). Each sample in a volume of 0.5 ml was eluted from a micro column containing 3% w/w deactivated silica gel using 10 ml of a 1:1 benzene: hexane mixture.

A Microtek-2000 gas chromatograph, equipped with a Coulson Conductivity Detector was used for preliminary screening for halogen in the sample extracts. Pyrex glass columns (1.83m X 4mm i.d.), packed with 5% OV-210 on 80/100 mesh Supelcoport, and 3% OV-1 on 70/80 mesh Chromosorb G were operated at 165° and 170° respectively.

Composited urine sample extracts were adjusted to 0.5 ml with pesticide grade hexane. Eight microliters

were injected into a Hewlett Packard 5700 A gas chromatograph containing a 1.22m x 2mm i.d. stainless steel column packed with 3% OV-1 on 80/100 mesh Gas Chrom Q. The initial column temperature was 150°, and the oven was programmed at 2°/min. to 210°. The injection port, transfer line and silicone membrane separator were kept at 200°, 220°, and 200° respectively. Helium flow rate past the membrane was 32 ml/min. Compounds transmitted through the membrane separator entered the ion source of a Hewlett Packard 5930A dodecapole mass spectrometer. Parameters of the mass spectrometer were as follows: ion source, 200°; mass filter, 110°; electron impact source at 70 eV; filament emission current, 250μ amps; target current, 220μ amps, scan rate 100 amu/sec from 45 - 450 amu. Ions were detected with a Bendix Continuous Dynode Electron Multiplier. Data were acquired, stored and plotted using the Hewlett Packard 5932A Data System.

RESULTS AND DISCUSSION

Recently HUTZINGER and co-workers (1972) demonstrated the metabolism of pure chlorobiphenyl isomers in pigeons and rats. The 4-chloro-, 4,4'-dichloro-, and 2,2',5,5'-tetrachlorobiphenyl isomers were metabolized to monohydroxy compounds. A dihydroxy monochloro metabolite was also reported. GARDNER, et al. (1973) fed 2,5,2',5'-tetrachlorobiphenyl to rabbits and identified 3-hydroxy-2,5,2',5'-tetrachlorobiphenyl, 4-hydroxy-2,5,2',5'-tetrachlorobiphenyl and trans-3,4-dihydro-3,4-dihydroxy-2,5,2',5-tetrachlorobiphenyl in urine samples.

In this study we have identified at least six hydroxylated biphenyl metabolites (Table I). Five of these compounds were found in both treatment groups. Of the three dihydroxylated metabolites, two were previously unreported. These compounds, a dichlorodihydroxy and a trichlorodihydroxy biphenyl, were found in both groups of animals. The only significant difference noted between the two treatment groups was the absence of a tetrachlorodihydroxy biphenyl in Aroclor 1242.

Significant quantities of hydroxylated chlorobenzenes were found in the urine extracts of both treatment groups (Table II). The source of these metabolites has not been determined but there is reason to suspect that they may have arisen from hexachlorobenzene which was found in the adipose tissue samples of the experimental animals.

TABLE I

PCB Metabolites Found in Urine of Rats Fed Aroclor 1016 or Aroclor 1242 (as Methyl Ether Derivatives)

Composition	Molecular Ion (M^+)	Aroclor 1016	Aroclor 1242
$C_{12}H_7Cl_2OCH_3$	252	yes	yes
$C_{12}H_6Cl_2(OCH_3)_2$	282	yes	yes
$C_{12}H_6Cl_3OCH_3$	286	yes	yes
$C_{12}H_5Cl_3(OCH_3)_2$	316	yes	yes
$C_{12}H_5Cl_4OCH_3$	320	yes	yes
$C_{12}H_4Cl_4(OCH_3)_2$	350	yes	no

TABLE II

Phenolic Metabolites Found in Urine of Rats Fed Aroclor 1016 or Aroclor 1242 (as Methyl Ether Derivatives)

Composition	Molecular Ion (M^+)
$C_6Cl_5OCH_3$	278
$C_6HCl_4OCH_3$	244
$C_6Cl_4(OCH_3)_2$	274

Quantitative estimation and exact identification of positional isomers of the observed metabolites was precluded because of the lack of authentic standards. However, GARDNER, et al. (1973) established positional identity for a specific tetrachlorohydroxylated biphenyl. By analogy, based on GLC retention and MS fragmentation data, it appears that more than one isomer of some of the hydroxylated biphenyls were present in these samples. Total ion current reconstructed chromatograms are presented in Figures 1 and 2.

Assignment of the molecular formulae of the observed metabolites was based on numerous mass spectra obtained during GLC separation for each component. In

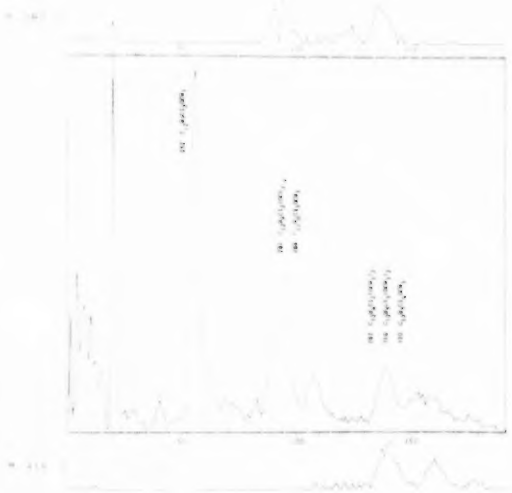

Figure 1. Reconstructed chromatograms of urine extracts from rats fed a diet of Aroclor 1242. 3% OV-1 column programed from 150° to 210° at 2° per minute.

Figure 2. Reconstructed chromatograms of urine extracts from rats fed a diet of Aroclor 1016. 3% OV-1 column programed from 150° to 210° at 2° per minute.

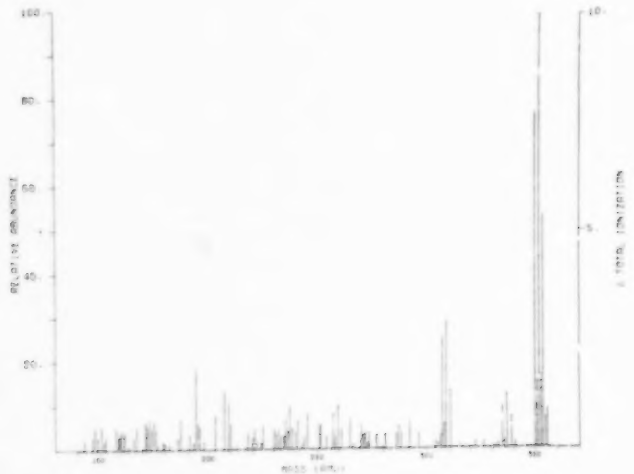

Figure 3. Mass spectrum of a methylated dihydroxytetrachlorobiphenyl (M^+=350) metabolite found in urine of rats fed a diet of Aroclor 1016.

a few instances, overlap of two or more components yielded spectra with more than one molecular ion.

The structural assignment of the methoxy derivatives of polychlorinated biphenyls rests upon the following: (1) The presence of intense molecular ions of the correct masses, (2) The correct chlorine "isotope clusters", to establish the number of chlorine substituents and (3) rational fragments, yielding the correct mass ions and appropriate chlorine "isotope clusters" for the fragments.

Figure 3 illustrates the typical features of the mass spectra of the methylated ether derivatives of the phenolic PCB metabolites. The intense molecular ion at m/e 350 and four chlorine "isotope cluster" are consistent with the assigned composition of a dimethoxytetrachlorobiphenyl, $C_{14}H_{10}O_2Cl_4$. Loss of methyl radical leads to the fragment at 335 amu containing four chlorines. Loss of methyl radical and of carbon monoxide from the aromatic ring, produces the largest fragment of M^+-43 with four chlorines at 307 amu. The loss of methyl radical and exclusion of carbon monoxide from aromatic rings is well documented for methoxy and dimethoxy aromatic ethers (BUDZIKIEWICZ, et al. 1967).

SUMMARY

Several mono- and dihydroxy metabolites of di-, tri, and tetrachlorobiphenyl have been identified in the urine of rats fed prolonged diets of Aroclor 1016 or Aroclor 1242. Combined gas chromatography-mass spectrometry was used for characterization of the metabolic products.

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The Non-Toxicity of Urine from Rats Poisoned With 1080

By **T. B. Gaines,** Biologist, **W. T. Sumerford,** Chemist, and **W. J. Hayes, Jr.,** Senior Assistant Surgeon, Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia

THE problem of whether rats poisoned by 1080 (sodium fluoroacetate) may excrete the poison or some toxic metabolic product in the urine has been mentioned earlier but, apparently, not studied. If such excretion were possible, food or other materials contaminated by 1080 poisoned rats during the relatively few hours which they survive, would carry an inestimably greater hazard for man than do materials contaminated by ordinary rat urine.

The problem was first approached chemically. Normal rat urines to which known amounts of sodium fluoroacetate had been added were analyzed for the acetate ion by the Kruger-Tschirch method[1] and several modifications of this procedure. Fluoroacetates respond to the classical test giving a blue color of different shade, and the test is satisfactory for 1080. However, a reaction between lanthanum nitrate used in the test and one or more unidentified constituents of rat urine invalidated the test through interference. Separation of the fluoroacetate from interfering substances in the urine was not accomplished, at least in part because the importance of the problem did not justify extensive analytical experimentation.

Bio-Assays Used

Bio-assays were then conducted to determine whether rat urine interferes with the toxicity of 1080 as well as with its detection by chemical means. Compound 1080 was dissolved in normal rat urine at the rate of 2 mg./cc. This solution was then administered by stomach tube to 10 adult white rats at a dosage of 10 mg. of 1080 (or 5 cc. of urine) per kilogram of body weight. Ten control rats were treated with the same dosage of 1080 prepared as a water solution. All the rats in both groups died within a period of 4 days. The average survival time of the rats poisoned by 1080 in urine was no longer than the average for rats poisoned by 1080 in water. Unpoisoned controls receiving 5 ml. of water or of urine without 1080 showed no effects.

Finally, bio-assays were done to determine the toxicity of the urine of rats poisoned by 1080. Fifteen adult white rats were poisoned with 1080 in water given by stomach tube. A dosage of 10 mg./kg. was used which killed all of the 15 rats within 4 days. The 14 ml. of urine collected from the 15 poisoned rats was divided equally between 2 white rats, weighing 200 gm. each. Each rat received, by stomach tube, 2 doses of 3.5 ml. 30 minutes apart. The rats were observed at 30-minute intervals during the first 8 hours and twice daily thereafter for a period of one week. Both rats remained entirely normal, exhibiting no symptoms of poisoning. This test was repeated except that 15 ml. of urine was obtained from 1080-poisoned rats and, as a result, two white rats weighing 200 gm. received 2 doses of 3.75 ml. of urine each. No symptoms of poisoning were detected in the test rats.

Conclusions

It may be concluded that: (1) The Kruger-Tschirch method or its modifications are not suitable for the detection of 1080 in rat urine, although it is useful for analysis of sodium fluoroacetate in the absence of interfering substances. (2) The addition of rat urine does not interfere with the toxicity to rats of 1080. (3) Rats poisoned by 1080 do not excrete the toxicant or any toxic metabolic product in the urine to an extent demonstrable on rats as tested. (4) If any excretion of the toxicant or its end products does occur in the urine, the amount is very small, and the significance of contamination of food or other materials by the urine of 1080-poisoned rats is the same as the significance of contamination by the urine of unpoisoned rats.

1. Pharm. Ztg., 74: 1096, 1929

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Liver Metabolism of Anticholinesterase Compounds in Live Rats: Relation to Toxicity

THE insecticide 'Isolan' (1-isopropyl 3-methylpyrazolyl dimethylcarbamate) is more toxic to rats [illegible] dermal than by the oral route[1]. This finding is interesting, because the opposite is true of most compounds. It was not known whether the unexpected difference in the toxicity of 'Isolan' by the two routes was caused by detoxification in the intestinal lumen, poor absorption from the gastrointestinal tract, detoxification in the liver, unusually efficient percutaneous absorption, or some other mechanism.

It is generally recognized that the liver is the main site of metabolism of foreign compounds in mammals.

Compounds may enter the body through a variety of routes: the gastrointestinal tract, skin and respiratory system being the most important. Absorption by any of these routes involves either the lymph or the blood as a primary transport medium. The lymphatic circulation and the capillary blood networks of the skin and lung discharge into the systemic blood circulation; the blood is then passed through the heart and on throughout the body. The liver receives about 6·5 per cent of the cardiac output directly through the hepatic artery and another 21 per cent of the cardiac output less directly by way of the mesenteric arteries and portal venous circulation. In contrast, the blood capillary network of the gastrointestinal tract discharges directly into the liver via the hepatic portal venous system, and essentially 100 per cent of this blood is delivered into the liver. Thus (if one ignores the possibility of a compound being sequestered by a given tissue or capillary bed) virtually all of a compound absorbed by the gastrointestinal tract goes directly to the liver; whereas, if a compound is absorbed from any other site, only 27·5 per cent reaches the liver before being distributed in the general circulation.

To test the effect of a single pass through the liver, 'Isolan' was injected directly into the hepatic portal system (intestinal vein) in one group of rats and into the general circulation (femoral vein) in another group. Similar investigations were conducted with two organic phosphorus compounds—dichlorvos, a direct inhibitor of cholinesterase, and parathion, an indirect inhibitor. It is well known that parathion is converted to the highly toxic paraoxon in the liver, but it is presumably in the liver also that it is eventually detoxified.

Adult female Sherman strain rats were paired according to age and body-weight and prepared for infusion by cannulating the intestinal vein of one rat and the femoral vein of the other. Surgery for installation of the cannulae

was performed on the rats while under anaesthesia with methohexital sodium solution given by intraperitoneal injection at a dosage level of 80 mg/kg. The method of cannulation of both veins was the same as that described by Ross and Rappaport[2] for cannulation of mesenteric veins.

As soon as the cannulation was completed and the incision sutured, each rat was confined to an individual restraining cage[3]. The cannulae were each fitted over the tip of a 27-gauge hypodermic needle and attached to separate 5-ml. hypodermic syringes on a dual infusion pump. Each rat was infused with normal saline, at the same rate as the poison formulation was later infused, for at least 1 h or until both rats recovered from the anaesthesia if recovery took longer. Then the syringes of normal saline were replaced with syringes containing the poison, and the infusion of the poison was started simultaneously in both rats.

Preliminary investigations were conducted with each of the compounds in a deliberate effort to establish a concentration, infusion rate, and duration of infusion which would kill all the rats infused by one route and allow those infused by the other route to survive. Once these conditions were determined for a compound, all the rats infused with that compound were infused at the same concentration and rate and for the pre-determined period of time, unless they died earlier. Based on the results of the preliminary tests, the compounds (all technical grade) used in this investigation were formulated and infused as shown in Table 1.

Each rat was observed continuously during the infusion of poison, and a complete record kept of time of the onset and course of symptoms and of the time of death. Except for the earlier detection of facial muscle fasciculation in some of the rats, the first symptom of poisoning was light generalized muscle fasciculation. Consequently, the time of onset of the latter was indicated as the time of onset of symptoms. Rats which survived the infusion were held for observation until recovery from symptoms, but not necessarily until recovery from cholinesterase inhibition.

The toxicity of 'Isolan', dichlorvos, and parathion infused into the hepatic portal system (intestinal vein) compared with their toxicity when infused directly into

Table 1. Formulation of Compounds and Rate of Intravenous Infusion in Female Rats

Compound	Vehicle	Formulation Concentration of poison (mg/ml.)	Infusion rate (ml./rat/min)	Maximal period of infusion (min)
'Isolan'	Normal saline	1·0	0·0083	60
Dichlorvos	Modified 'Lipomul'*	12·5	0·0068	60
Parathion	Modified 'Lipomul'*	1·0	0·0068	75

* As described by Tuilner and Herts (ref. 4).

Table 2. Intravenous Toxicity of Pesticides infused into the Hepatic Portal System (Intestinal Vein) compared with their Toxicity when infused into the General Circulation (Femoral Vein) in Female Rats

Compound	Route of infusion	Body weight of rats (g)		Time to onset of symptoms (min)		Time to death (min)		Total dose (mg/kg)		Mortality (died/tested)
		Range	Mean ± S.E.	Range	Mean ± S.E.	Range	Mean ± S.E.	Range	Mean ± S.E.	
'Isolan'	Femoral vein	237–392	322 ± 21·8	10–15	13 ± 0·9	18–35	28 ± 2·5	0·44–1·00	0·73 ± 0·08	6/6
	Intestinal vein	255–368	311 ± 18·6	30–55*	48 ± 4·6	One died		1·35–1·95	1·63 ± 0·10	1/6
Dichlorvos	Femoral vein	292–410	346 ± 16·6	5–9	7 ± 0·1	17–31	21 ± 2·1	4·4–6·4	5·9 ± 0·29	6/6
	Intestinal vein	300–408	339 ± 16·9	14–29	24 ± 2·3	Survived		12·5–17·0	15·2 ± 0·71	0/6
Parathion	Femoral vein	270–336	305 ± 10·6	55–75†	64 ± 4·5	Survived†		1·52–1·89	1·69 ± 0·06	0/6
	Intestinal vein	280–354	315 ± 10·9	26–39	34 ± 1·9	57–75	65 ± 2·9	1·27–1·60	1·41 ± 0·05	6/6

* One rat showed no symptoms of poisoning.
† Three rats showed no symptoms of poisoning.

the general circulation (femoral vein) in rats is shown in Table 2. Based on time to onset of symptoms, time to death, and mortality in the poisoned rats, it is apparent that 'Isolan' and dichlorvos infused into the hepatic portal system undergo considerable detoxification during a single passage through the liver before they reach the general circulation. On the other hand, parathion infused into the hepatic portal system is more toxic than when infused by way of the femoral vein into the general circulation.

Time to onset of symptoms caused by 'Isolan' and dichlorvos was delayed about three times as long in rats infused into an intestinal vein as in those infused in the femoral vein. Six rats given a mean total dose of 0·73 mg/kg of 'Isolan' in the femoral vein were killed, but 5 of 6 rats given a mean total dose of 1·63 mg/kg by intestinal vein survived. A mean total dose of 5·0 mg/kg of dichlorvos killed 6 rats infused in the femoral vein, but 6 rats infused in an intestinal vein survived a mean total dose of 15·2 mg/kg.

The results for parathion presented in Table 2 indicate that the time to onset of symptoms in rats infused in the femoral vein was about twice as long as that for rats infused in an intestinal vein. However, 3 of the 6 rats infused in the femoral vein for 75 min never exhibited any signs of poisoning and none of the 6 rats died. All the 6 rats infused in an intestinal vein were killed in 57–75 min.

It is clear that efficient detoxification of 'Isolan' by the liver *in vivo* is one factor contributing to its relatively low toxicity by the oral as compared to the dermal route. However, one or more other factors must be important, for the detoxification of dichlorvos by the liver seems equally efficient, and yet dichlorvos is slightly more toxic by the oral than by the dermal route. Even though other techniques will have to be used to explain fully the pattern of toxicity of 'Isolan' when given by different routes, the technique presented in this report is a useful and relatively simple procedure for learning the net effect of the liver on the metabolism of chemicals in intact laboratory animals.

THOMAS B. GAINES
WAYLAND J. HAYES, JUN.
RALPH E. LINDER

Toxicology Section,
Technology Branch,
Communicable Disease Center,
U.S. Department of Health, Education and Welfare,
Atlanta, Georgia.

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